Effekte eines Synergy1-Fermentationüberstandes auf die Primär-und Sekundärprävention in Kolonkrebszellen in vitro

Dissertation

zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena

von
Umang Munjal
Master of Veterinary Science (M.V.Sc.)

geboren am 26.03.1977
in Hisar, Indien

Effects of Synergy1 Fermentation Supernatant on Primary and Secondary Chemoprevention in Colon Cancer Cells *in vitro*

Dissertation

for obtaining the degree of doctor rerum naturalium (Dr. rer. nat.) at the Faculty of Biology and Pharmacy, Friedrich-Schiller-University Jena

submitted by
Umang Munjal
Master of Veterinary Science (M.V.Sc.)

Born on 26.03.1977
at Hisar, India

Reviewers:

1. _____

   _____

   _____

2. _____

   _____

   _____

3. _____

   _____

   _____

Day of the public defence:

Dedicated to Priya

Contents

I. List of figures

II. List of tables

III. List of abbreviations

%	Percentage
/	Per
<	Less than
>	Greater than
°C	Degree centigrade
µm	Micromole
ACF	Aberrant crypt foci
AMC	7- amino-4-methoxy coumarin
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
BB12	Bifidobacterium lactis Bb12
BSA	Bovine serum albumin
Ca	Calcium
CAT	Catalase
CDNB	1-chloro-2 4-dinitrobenzene
cm^2	Centimeter square
CO_2	Carbondioxide
C_t	Threshold cycle
CTB	Cell titer blue
DAPI	4', 6-diamidino-2-phenylindole dihydrochloride
DCA	Desoxycholic acid
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTPs	Desoxyribose nucleoside phosphate
DP	Degree of polymerization
DR4	TRAIL Receptor 1
DR5	TRAIL Receptor 2
DTT	Dithioreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamintetraacetic acid
EPIC	European prospective investigation
Ex/Em	Excitation /Emission
FAP	Familial adenomatous polyposis
FB	Faecal blank
G	Gram
GAPDH	Glyceraldehyde phosphate dehydrogenase
GST	Glutathione S Transferase
GSTs	Glutathione-S-Transferases
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDACIs	Histone deacetylase inhibitors
HNE	4-hydroxy-nonenal

HNPCC	Hereditary non polyposis colorectal cancer
HPFS	Health Professional's Follow up study
HRP	Horse raddish peroxidase
HSPA5	Heat shock protein5
HT29	Highly transformed adenocarcinoma cells
kDa	Kilodalton
K*ras*	Kirsten rat sarcoma
K-ras	Kirsten ras oncogene
LGG	Lactobacillus rhamnosus GG
LMA	Low Melting Agarose
LT97	Preneoplastic adenoma cells
m	Meter
M	Molar
M	Molar (Mol/l)
mA	Mili Ampere
Mg	Magnesium
mg	Milligram
min	Minute
min	Minute
ml	Milliliter
mRNA	Messenger ribonucleic acid
MT2A	Metallotheoneine 2A
NaOH	Sodium hydroxide
NFkB	Nuclear factor kappa B
NHS	Nurses Health Study
O_2^-	Superoxide anions
OH^-	Hydroxyl radicals
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethane sulfonylfluoride
RIN	RNA integrity number
rpm	Revolutions per min
SCFA	Short chain fatty acids
SD	Standard deviation
SD	Standard deviation
SDS	Sodiumdodecylsulfate
SFM	Synthetic fermentation mixture
SFS	Synergy1 fermentation supernatant
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TGF-ß	Transforming growth factor ß
UV	Ultraviolet
V	Voltage
v/v	Volume per volume
WCRF	World Cancer Research Fund

1 Introduction

1.1 Nutrition and Cancer

Nutrition and health are closely connected. Numerous plant foods or physiologically active ingredients derived from plants have been investigated for their role in disease prevention and health [Gwyn et al., 2002]. The main reason for nutrition–dependent diseases in our society, however, is over nutrition and malnutrition characterized by high energy uptake and an unbalanced diet ("western style diet"). Imbalanced nutrition results in a higher risk for developing diseases such as arteriosclerosis, cancer, obesity and diabetes. Within this list, cancer is of special importance, because not only does it account for one quarter to one third of deaths in countries with western style diet but also has dramatic consequences for the affected individuals [Berlau et al., 2004]. The most frequent type of cancer in this context is colorectal cancer with more than 100,0000 new patients and 500,000 deaths annually worldwide [Dirk Schrijvers, 2008].

1.2 Colorectal cancer: Incidence and prevalence

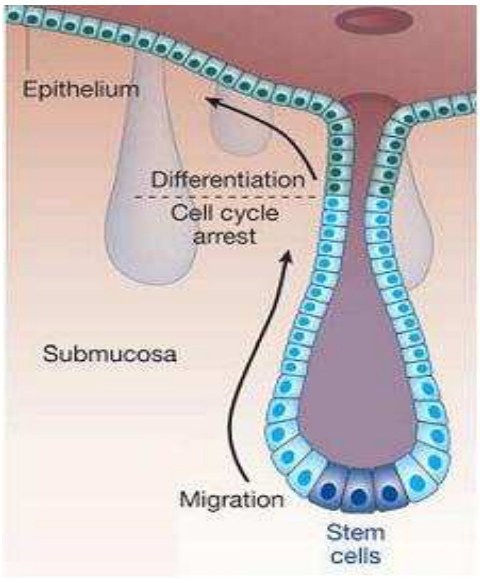
Cancers of colon and rectum are the third most common type of cancers worldwide. Around 1 million cases were recorded in 2002, accounting for around 9 percent of all types of cancer [WCRF/AICR, 2007]. There is no clear trend in global age-adjusted rates of colorectal cancer. In general, the age standardized incidence rate of colorectal carcinoma is very high in Western Europe, Australia and New Zealand, whereas it is very low in India and Africa [Parkin and Muir, 1992;Tamakoshi et al., 2001]. There has however been a rapid increase in rates in high income countries that have recently made the transition from a relatively low income economy such as Japan, Singapore and Eastern European countries [WCRF/AICR, 2007]. Rates have at least

doubled in many of these countries since the mid 1970s [Parkin and Muir, 1992]. Studies have also shown that risk of colorectal cancer changes in migrants after a period of 20-30 years in a high risk country, and that the risk of developing colorectal cancer may decrease after migration from a high to a lower risk country [Schottenfeld and Beebe-Dimmer, 2005]. For example, the incidence increased for Japanese migrants after moving to Hawaii or from Southern Europe to Australia [McMichael et al., 1980; Schottenfeld and Beebe-Dimmer, 2005]. In Europe, colorectal cancer is the most common form of cancer, too, with more than 300,000 cases a year even more than lung cancer. It is the second-leading cause of cancer deaths in European men and women (www.oncolink.com).

The lifetime colorectal cancer risk in general population is reported to be approximately 5 %, with individual risk increasing significantly with age [Pisani et al., 1990]. Additionally, family history of colorectal cancer increases the risk and there are several rare genetic syndromes which account for about 5 % of all cases of colorectal cancer [Potter, 1999].

1.2.1 Molecular mechanisms of colorectal carcinogenesis

The intestinal tract consists of the small intestine (duodenum, jejunum and ileum) and the large intestine (caecum, colon and rectum). The absorptive surface of the small intestine is covered with villi (finger like outgrowths) which increase the amount of surface area available for absorption of digested food. The colonic epithelium in the large intestine has larger crypts. Crypts are anatomical structures that are narrow and deep invaginations which secrete various enzymes. Proliferative stem and precursor cells occupy the bottom two-thirds of the crypts, whereas differentiated cells constitute the surface epithelium and top third of the crypts (Figure 1).



Putative stem cells (dark blue) populate the bottom of the crypt. Proliferating progenitor cells (light blue) occupy two thirds of the crypt. Differentiated cells make up the rest one third and surface of the epithelium, which in contrast to the small intestine is a flat surface [Reya and Clevers, 2005].

Figure 1 Tissue anatomy of human large intestine

Colorectal cancer (CRC) is usually observed in one of two specific patterns; sporadic and inherited. The two most common inherited syndromes associated with an increased risk of CRC are familial adenomatous polyposis coli (FAP) and hereditary non polyposis colorectal cancer (HNPCC) also called Lynch Syndrome. FAP is a rare autosomal dominant syndrome and least understood pattern of colon cancer development [de and Fernando, 1998]. A germline mutation in the tumour suppressor gene for adenomatous polyposis coli (APC) results in FAP [Kinzler and Vogelstein, 1996]. Almost 20 % of cases occur in people who have a family history of colorectal cancer [Lynch and de la, 2003]. HNPCC is an inherited autosomal dominant syndrome [Jass et al., 1994]. Specific genetic mutations have been identified as the cause of HNPCC, these mutations are estimated to account for only 5-10 % of colorectal cancer cases overall. HNPCC is characterised by inactivated DNA mismatch repair (by inherited or acquired mutation or methylation) that results in microsatellite instability in the tumours [Lynch and Lynch, 2000]. People with FAP develop a large number of adenomas at a relatively young age; if left untreated, nearly

all will develop colorectal cancer by the time they reach the 40s whereas, people develop HNPCC in their mid 40s and HNPCC further increases the risk of other gastrointestinal cancers [Weitz et al., 2005]. Other colon tumours associated with predisposing factors are related to inflammatory bowel diseases. However, the greatest numbers of colorectal cancers develop either sporadically or are caused by external factors [Berlau et al., 2004] (Figure 2). The external factors can cause genetic alterations or modulate the processes for the development of cancer namely initiation, promotion and progression.

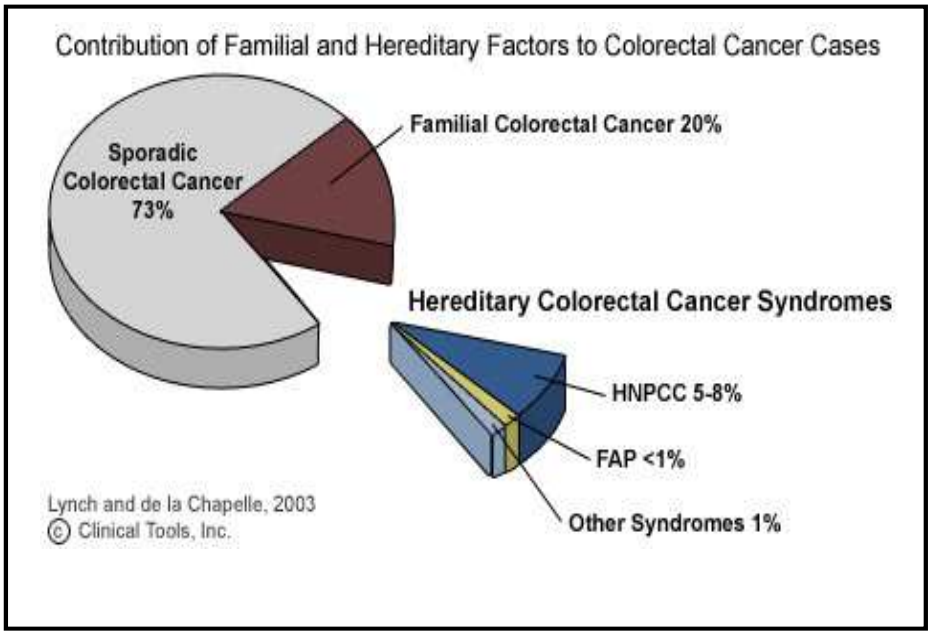


Figure 2 Causes of Colorectal Cancer
(www.images1.clinicaltools.com/images/gene/crcpiechart)

The pathogenic pathway leading to the so-called adenoma-carcinoma sequence was first described by Hill and colleagues [Hill et al., 1978]. According to them mutations in at least four or five genes are required for formation of a malignant tumour (Figure 3). The first events are mutations in the gene APC (adenomatous polyposis coli) in both chromosomes. APC is a human gene that is classified as tumour suppressor gene

and prevents the uncontrolled growth of cells that may result in cancerous tumours [Fodde et al., 2001]. When a mutation eliminates the function of APC gene, the cells are immediately launched on the pathway towards malignancy. Loss or mutation of APC thus induces polyp formation as a result of loss of orderly cell replication, adhesion and migration. This is followed by mutation in the oncogene K-ras and further mutation of the tumour suppressor genes SMAD4 and TP53. Other genetic events also play a role, for example modulation of DNA methylation in CpG sequences of the promoter regions of tumour-suppressor and DNA repair genes, leading to inactivation, or DNA amplification as a mechanism of oncogene activation [Kinzler and Vogelstein, 1996]. These genetic alterations are associated with the development of preneoplastic lesions (aberrant crypt foci, polyps, adenomas) which can develop into carcinomas [Vogelstein et al., 1989]. It has now become clear that in HNPCC, in addition to the above mentioned genes some other genes e.g. Transforming growth factor ß (TGF ß) or Bax (involved into apoptosis) are also damaged [Rampino et al., 1997]. Furthermore, ulcerative colitis also predisposes to the risk of colorectal cancer. Although this disease is a minor contributor to the overall population burden of colorectal cancer, individuals with ulcerative colitis have about a 20-fold excess risk [Brentnall et al., 1996].

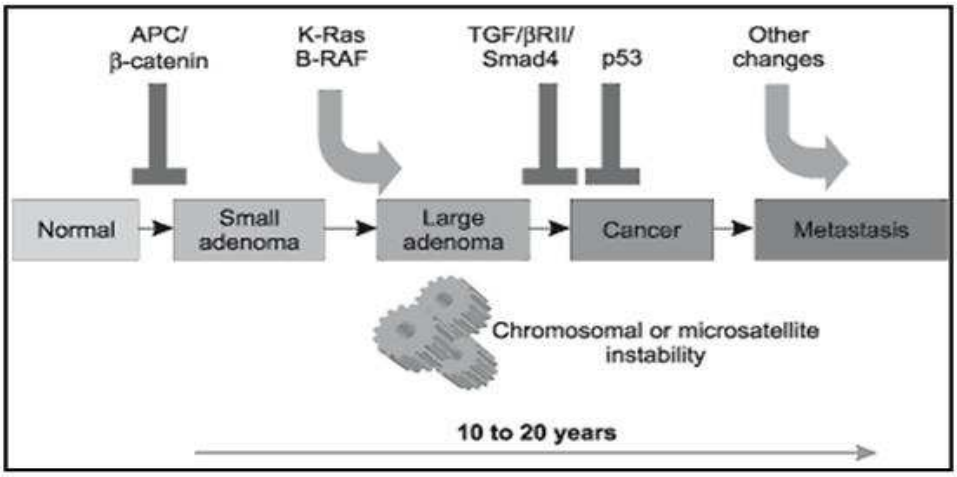


Figure 3 Molecular Mechanisms of Colorectal Carcinogenesis

APC, Adenomatous polyposis coli, K ras, Kirsten rat sarcoma; SMAD4, mothers against decapentaplegic, drosophila, homolog of 4; TGFBR2, transforming growth factor-β receptor, type 2; TP53, tumour protein p53; B-RAF, v-raf murine sarcoma viral oncogene homolog B1 [Tejpar and Van, 2003]

1.2.2 Risk factors for colorectal cancer

Epidemiological studies have suggested that the incidence of colon cancer could be increased by composition of diet [Keown-Eyssen and Bright-See, 1984; Hill, 1997]. However, the mechanisms which increase the risk are not clear. It has been suggested that DNA damage in the human colonic cells can be caused by specific food ingredients or their metabolic products for e.g. red meat, processed meat and substantial consumption of alcoholic drinks [WCRF/AICR, 2007]. High meat consumption for instance leads to higher levels of amino compounds, nitrosamines, an altered gut flora and changed enzyme patterns. These compounds may be either cytotoxic or genotoxic themselves or increase the formation of genotoxic compounds in the gut lumen. Furthermore, low folate uptake, combined with high alcohol uptake, might pose another risk to colorectal cancer as lack of folate leads indirectly to physiologically disturbed DNA methylation, which might inhibit transcription of tumour suppressor genes or increase transcription of proto-oncogenes [Berlau et al., 2004]. Moreover, it has been shown that physically active persons with a lower body mass index are at less risk of cancer [Kinzler and Vogelstein, 1996].

Nutritional factors not only affect colorectal carcinogenesis as a result of the direct activity of individual compounds, but also act indirectly by enhancing the lipid peroxidation. Dietary intake of polyunsaturated fats represents a major source of lipid hydroperoxides in the intestinal lumen [Bartoli et al., 1993]. In addition to nutritional factors, radical structures such as superoxide anions (O_2^-) and hydroxyl radicals (OH^-) are generated in biological systems not only by extrinsic factors as, for example,

visible light or ionising radiation, but also by intrinsic factors like by-products emerging during oxygen metabolism or cellular redox reactions [Oberreuther-Moschner et al., 2005]. These radicals can react directly with DNA resulting in oxidized bases and, ultimately, mutations after replication [Berlau et al., 2004] which in turn increases the risk of degenerative diseases such as colorectal cancer. Unfortunately, our knowledge of the specific substances which are actually responsible for molecular alterations in cell transformation is still fragmentary, and adequate toxicological assessment of relevant endogenous risk factors is usually still missing.

1.3 Chemoprevention of colorectal cancer

1.3.1 Overview of chemoprevention

Cancer chemoprevention, i.e. the use of chemicals or dietary components to block, inhibit or reverse the development of cancer in normal or preneoplastic tissue, is considered one of the most promising areas in current cancer research. There are currently three strategies for cancer prevention [Kanduc et al., 2003]:

I. Primary chemoprevention: removal or neutralization of etiological agents,

II. Secondary chemoprevention: interference with either the tumour promoting metabolic mechanisms associated with particular etiologic agents or with the responses of the target cells or tissue to an activated form of an etiological agent,

III. Tertiary chemoprevention: interrupting the sequence of events during the cellular evolution from precancer to cancer.

Much of the work on the anticarcinogenic properties of naturally occurring compounds has been carried out by Wattenberg and his coworkers [Wattenberg, 1992]. According to them, anticarcinogens are subdivided into two major classes

defined as “blocking agents” and “suppressing agents” (Figure 4). Blocking agents are typically compounds which have been thought to prevent initiation, either by inhibiting the formation of carcinogens from precursor compounds, or by preventing the active carcinogenic species from acting upon its cellular target. In contrast, suppressing agents are thought to act by preventing the progression of initiated cells to fully transformed tumour cells, e.g. by inhibition of cellular proliferation or induction of apoptosis.

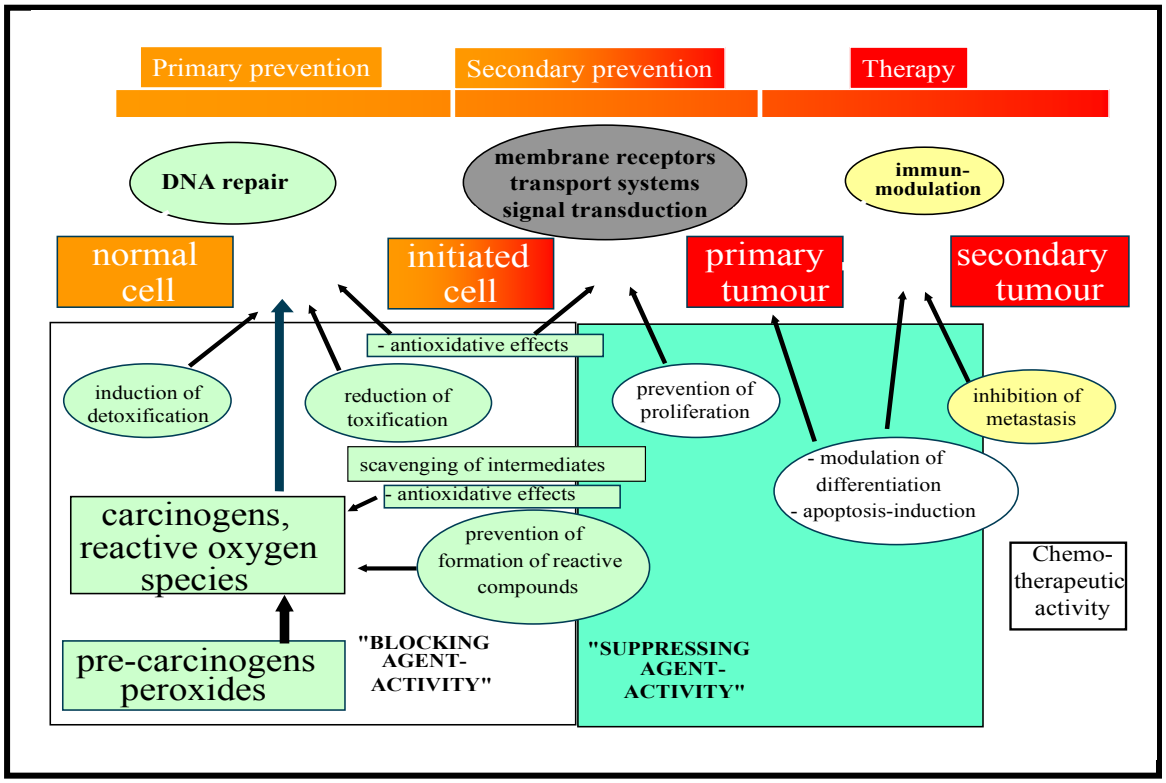


Figure 4 Mechanisms whereby protective factors may inhibit Carcinogenic process. (modified according to Johnson et al., 1994)

1.3.2 Diet and chemoprevention

The elucidation of molecular sites of action for dietary components is fundamental for the development of effective prevention strategies. Studies indicate that dietary constituents can influence various genetic and epigenetic events that determine cellular metabolism, differentiation and apoptosis [Bradlow et al., 1999; Knowles and

Milner, 2000]. Compounds encompassing such diverse categories as carotenoids, flavonoids, glucosinolates, isothiocynates and fermentable dietary fibres have been found to reduce experimentally induced cancers. Numerous reviews highlighting the benefits (and possible risks) of these and other bioactive food components have been published [Abdulla and Gruber, 2000; Pool-Zobel, 2005]. These chemopreventive compounds induce biological responses by serving as an antioxidant, promoting the activity of detoxification enzymes, blocking carcinogen formation, shifting hormonal homeostasis, retarding cell division and inducing apoptosis [Milner, 2002].

Among various chemopreventive compounds studied so far, vitamin A has been linked to differentiation [Milner, 2002], whereas vitamin D inhibits the accumulation of ß-catenin by facilitating its degradation [Murillo and Mehta, 2005]. The reduced ß-catenin causes a decrease in its transcriptional activity that stimulates the expression of oncogenes such as c-myc. Moreover, vitamin E and C and the mineral selenium can act as antioxidants [Bingham, 1996]. Furthermore, literature involving the inhibition of inflammatory processes by compounds such as butyrate [Hamer et al., 2008], n-3 fatty acids [Brink et al., 2004] and dietary fibres [Roberfroid, 1993] is increasing.

1.4 Dietary fibres as potential chemoprotective substances

1.4.1 Dietary fibres

Since the original description of dietary fibres as remnants of edible plant cell polysaccharides and associated substances resistant to hydrolysis by human alimentary enzymes [Trowell, 1976], the definition has been extensively debated. The American association of cereal chemists defined dietary fibres as the remnants of edible parts of plant and analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the human large intestine [Jonathan, 2003]. Furthermore, it also defines oligosaccharides

as being dietary fibres. Therefore, "dietary fibres" consist of a great variety of food components mainly cell wall fractions as well as some storage carbohydrates.

During their passage through the upper intestine, dietary fibres influence the digestion process through several mechanisms. It stimulates bacterial growth, and together with water binding to residual unfermented fibre, it leads to an increase in stool weight, dilution of colonic contents and faster transit time through the large gut [Cummings, 1981; Phillips et al., 1995]. Faecal bulking as well as reduced transit times has been associated with the protection against colorectal cancer by decreasing the probability of colonic carcinogen exposure [Ferguson et al., 2000].

Additionally, dietary fibres entering the large bowel stimulate anaerobic fermentation, leading to the production of short chain fatty acids (SCFA), acetate, propionate, and butyrate and an increase in microbial cell mass [Bingham, 1996]. Of these SCFA, butyrate is physiologically relevant to the colonic epithelium by serving as a principle energy source. Interest in its role as possible chemoprotective agent has arisen from its properties to inhibit proliferation and induce apoptosis of cancer cells *in vitro* [Kruh, 1982]. Next to SCFA there are still other fermentation products that arise mainly from a wide variety of plant ingredients for example phytic acid, hydrocinnamic acid and in particular ferulic acid from wheat bran. These compounds have also been shown to have diverse biological activities such as scavenging of radicals [Ogiwara et al., 2002] or acting anti inflammatory and anti carcinogenic in rat colon [Ozaki, 1992].

1.4.2 Epidemiological and clinical data for relationship between dietary fibres and colorectal cancer

The potential importance of dietary fibre intake for prevention of colorectal cancer was suggested more than three decades ago by Burkitt, who related the low incidence of colon cancer in parts of Africa to the local diets, which were high in plant foods

and dietary fibres [Burkitt, 1969]. Burkitt hypothesized that high fibre intake results in high stool bulk, which reduces stool transit time and thus lowers exposure of colonic epithelium to potential carcinogens. A combined analysis of 13 [Howe et al., 1992] case control studies as well as a meta analysis of 16 case control studies [Trock et al., 1990] indicated an inverse association between fibre intake and colorectal cancer. In contrast, 10 prospective studies including initial analysis from the Nurses Health Study (NHS) [Fuchs et al., 1999] and Health Professional's Follow up study (HPFS) [Giovannucci et al., 1994] have largely failed to support this association. Most recently the European Prospective Investigation into Cancer and Nutrition (EPIC) including 1065 incident cases of colorectal cancer among 519,978 individuals followed for an average of 4.5 years described an inverse link between fibre intake and colorectal cancer incidence [Bingham et al., 2003].

1.4.3 Short chain fatty acids as products of gut fermentation of dietary fibres

The physiology of the fermentation process of dietary fibre has been reviewed in depth [Scheppach et al., 1995]. The major products of fermentation of dietary fibre metabolism are SCFAs, the gases H_2 and CO_2 and bacterial cell mass [Cummings and Macfarlane, 2002]. SCFA are produced in the proximal colon in an average molar ratio of acetate: propionate: butyrate 60:20:20 mmol/l [Wong et al., 2006]. This ratio, however, is fairly constant, although alterations in production and absorption may occur with the kind of substrate fermented [Cummings et al., 1987]. Much has been written about SCFA production in the hind gut and the differing metabolic significance of the individual SCFA [Cummings and Macfarlane, 2002].

Among SCFA, butyrate serves as an energy yielding substrate in colonocytes and additionally affects several cellular functions like induction of apoptosis, suppression of cell proliferation, anti-inflammatory actions, up regulation of immunosurveillance

and down regulation of angiogenesis [Williams et al., 2003]. Butyrate seems to be of essential importance for the metabolic welfare of normal intestinal epithelia where it prevents mucosal atrophy [Wachtershauser and Stein, 2000]. Interest in its role as a possible protective agent has arisen from its anti-proliferative and pro-apoptotic effects on colon cells [Hague and Paraskeva, 1995]. Moreover, literature depicts that butyrate also induces Glutathione S Transferases (GSTs) detoxifying enzymes that provide defence against carcinogens [Pool-Zobel et al., 2005]. Butyrate was also found to modulate *c-myc* expression by post translational degradation of the mRNA [Souleimani and Asselin, 1993]. C-myc belongs to the family of proto-oncogenes and their gene products are involved in cell growth [Rochlitz et al., 1996]. Propionate and acetate are metabolized less than butyrate and are transported to liver [Cook and Sellin, 1998]. Propionate is substrate for hepatic gluconeogenesis and has been reported to inhibit cholesterol synthesis in hepatic tissue whereas acetate is utilized in the synthesis of long chain fatty acids, glutamine and betahydroxybutyrate [Rombeau and Kripke, 1990].

1.5 Inulin type fructans as potential dietary fibres

Among various dietary fibres inulin and oligofructose have been subjected to extensive research [Pool-Zobel, 2005; Roberfroid, 2005]. Inulin type fructans are the natural constituents of a wide range of common vegetables and fruits such as onion and garlic. The estimated average daily intake per capita of inulin-type fructans from natural sources is 3-11 g in Western Europe and 1-4 g in North America [Moshfegh et al., 1999]. Typically, inulin has a degree of polymerization (DP) between 3 and 60 (average DP is approximately 12) and is produced industrially from chicory roots by hot water extraction, followed by refining and spray drying. Structurally, inulin type fructans are polydisperse carbohydrates consisting mainly of ß (2-1) fructosyl fructose links. This linkage cannot be hydrolysed by pancreatic or brush border digestive

enzymes. Therefore these fructans reach the colon undigested, where they are fermented by *Bifidobacterium* spp. and other lactic acid-producing bacteria to yield SCFA and gases. Therefore, they are classified as "dietary fibres" (oligo and polysaccharides) that resist both hydrolysis by mammalian digestive enzymes and absorption in the small intestine but are, at least partly, hydrolyzed and fermented by the colonic flora [Roberfroid, 2005].

Synergy1, a commercially available mixture of inulin enriched with oligofructose (Orafti Belgium) is a prebiotic dietary fibre that has been specifically formulated to improve digestive health (Figure 5). *In vitro* fermentation experiments revealed that molecules with a DP >10 like inulin are fermented, on an average, half as quickly as molecules with a DP of < 10 like oligofructose [Ten Bruggencate et al., 2004]. Rapid fermentation of oligofructose by the intestinal microflora may lead to high luminal concentrations of organic acids which in turn may induce damage to the mucosal barrier [Ten Bruggencate et al., 2004]. Therefore in order to decrease the rapid fermentation of oligofructoses, Synergy1 is enriched with inulin. Thus, the longer chain fructan is fermented at a slower and more selective rate which maintains the metabolic activity of the gut flora for a longer period of time [Van Loo et al., 2005]

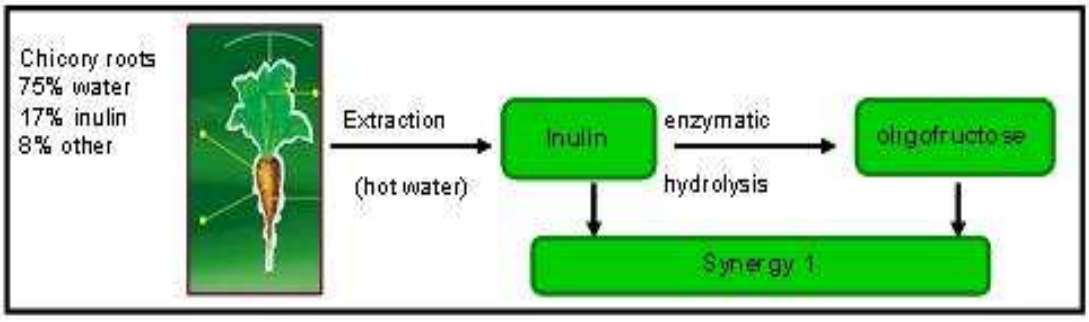


Figure 5 Preparation of Synergy1 (modified according to www.orafti.com)
The inulin is extracted from chicory roots using hot water. Oligofructose is then obtained through partial enzymatic hydrolysis. Synergy1 is a composition of oligofructose and inulin.

1.5.1 Biological activities of Synergy1

In experimental animals a large number of publications demonstrate that inulin type fructans significantly increase mineral absorption, essentially Ca and Mg [Weaver and Liebman, 2002]. In a study aimed at comparing oligofructose, inulin and Synergy1, Coudray *et al.* have shown that the latter product was more active than oligofructose or inulin alone in enhancing Ca and Mg absorption [Coudray et al., 2003].

Modulation of either the digestion/absorption or the metabolism of lipids is another physiological effect of inulin-type fructans that influence triglyceridaemia and cholestrolaemia as well as the distribution of lipids between the different lipoproteins in favour of a more beneficial health pattern [Delzenne et al., 2002].

Moreover, inulin type fructans beneficially affect the intestinal immune system by targeting gut-associated lymphoid tissues and especially Payer's patches, and consequently have been shown to decrease the risk of diseases related to dysfunction of gastrointestinal defense functions [Roberfroid, 1993].

1.5.2 *In vitro* and *in vivo* studies of inulin type fructans in relation to colon cancer

A number of different experimental studies have shown that the fermentation products of inulin contribute to colon cancer-preventing properties, as has been summarized in two recent reviews [Pool-Zobel, 2005; Roberfroid, 2007]. Regarding the risk of colon cancer it has been shown that gut fermentation products of inulin-type fructans beneficially modulate markers of tumour progression in human colon tumour cells [Beyer-Sehlmeyer et al., 2003; Klinder et al., 2004a]. *In vivo* studies by Buddington et al evaluated chicory fructans for their potential inhibitory properties against total ACF formation in the colon of rats and mice [Buddington et al., 2002]. These studies were further supported by Poulsen *et al.* who also observed a significant inhibition in the number of ACF in rats after feeding inulin [Poulsen et al., 2002]. Not

only do these fermentation products reduce the number and the size of lesions but also they reduce the risk of progression of these lesions towards malignancy. An *in vitro* study by Klinder *et al.* also reports distinct functions of inulin in inhibiting the growth and metastasis in colon tumour cells [Klinder et al., 2004a]. All of these findings suggest potential colon tumour inhibitory properties of these fructans. The mechanisms proposed to explain these beneficial effects include changes in the composition and/or activity of colonic microflora (prebiotic effect), and in the composition of the SCFA pool and especially an increased relative proportion of butyrate as a result of anaerobic fermentation. Therefore, these studies indicate that fermentation supernatants derived from Synergy1 impair growth, survival, and progression of human tumour cell lines, all mechanisms that are associated with suppressing activity and secondary cancer prevention. Moreover, these fructans strengthen and stimulate gastrointestinal defense functions and especially intestinal immunity, two effects that certainly improve resistance to cancer development [Van Loo et al., 2005].

In a human intervention study, the synbiotic treatment [(oligofructose-enriched inulin (Synergy1) + *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium lactis* Bb12 (BB12)] resulted in a reduction of DNA damaging capacity of faecal water in polyp patients [Rafter et al., 2007]. The modulation of DNA damage reflects reasonably well the modulated exposure to genotoxic compounds that cause the damage and hints towards the "blocking agent" activity of these fructans. Another human study detected a decline of toxic bile acids in faecal samples after a 3 months intervention with oligofructoses [Boutron-Ruault et al., 2005].

Thus, inulin type fructans exert several effects on the gastrointestinal tract, but the precise mechanisms for its probable protective role are still not clearly understood. Therefore, it was of interest to explore the biological effects of inulin type fructans and their metabolites in terms of colon cancer prevention. In the present study the

fermentation sample from Synergy1 was tested *in vitro* using human adenoma and carcinoma cells (representing an early and a late stage of carcinogenesis). This study presents an experimental approach to analyze the biological activities of dietary fibres and their resulting complex fermentation products. The results were expected to enhance our understanding of the chemo-preventive properties of dietary fibres in terms of reducing colorectal cancer risk.

2 Objectives of the study

To elucidate chemopreventive mechanisms of dietary fibres investigation strategies are needed to characterise not only the fibre itself, but rather the metabolites produced after fermentation by the gut flora. The formed products have the potential to interact with cells of the gut mucosa and elicit a number of different biological activities. Therefore, the aim of this study was to find possible chemo-preventive effects of Synergy1, a prebiotic dietary fibre and its metabolic products *in vitro* in human colon cancer cell lines. Since chemoprevention means preventing non transformed cells from being initiated (primary chemoprevention) or preventing further growth of already transformed cells (secondary chemoprevention), a series of experiments were performed in human colon tumour cells to elucidate possible chemoprotective mechanisms [growth inhibition, anti genotoxic and apoptosis inducing mechanisms of Synergy1 fermentation supernatant (SFS)].

Therefore, two different colon cancer cell lines were used namely LT97 (preneoplastic adenoma cells) and HT29 (highly transformed adenocarcinoma cells) to address the following questions:

- Does fermentation of Synergy1 result in increased production of metabolites mainly SCFA and reduction of toxic secondary bile acids? **(Published)**
- Does incubation with SFS modulate the growth of both cell lines, if yes; whether the modulation of cell growth is due to the concentration of metabolites? **(Published)**
- Does incubation with SFS protect the DNA from oxidative stress which in turn could be a reflection of inhibitory activities related to inhibition of tumour initiation?

- Is the modulation of oxidative stress by SFS due to the ability to modulate mRNA expression of phase II genes and corresponding enzymes of biotransformation?
- Does incubation with SFS induce PARP cleavage; a marker of apoptosis **(Published)** and modulate the activity of apoptotic enzymes mainly caspases?
- Are chemo-preventive mechanisms of SFS also involved in modulation of genes relevant to apoptosis (secondary chemoprevention)?
- Are apoptosis inducing activities of fermentation supernatant similar to a synthetic fermentation mixture (SFM) mimicking the SFS in the amount of SCFA and desoxycholic acid (DCA), a secondary bile acid or due to presence of other substances?

3 Materials and Methods

3.1 Materials

3.1.1 Cell models

LT97 cells

The human colon adenoma cell line LT97 was a kind gift from Professor Brigitte Marian (Institute for Cancer Research, University of Vienna, Austria) who established it from colon microadenomas of a patient with familial adenomatous polyposis [Richter et al., 2002]. These cells represent an early stage of colorectal cancer.

HT29 cells

The HT29 cell line that had been established by Fogh and Trempe from a colon adenocarcinoma of a Caucasian female [Fogh, 1986] was originally purchased from the American tissue culture collection (Rockville, MD). When these cells are grown under standard conditions, they display an undifferentiated phenotype [Cohen et al., 1999]. HT29 cells are highly transformed and represent a late stage of colorectal cancer.

3.1.2 Test substances

The carbohydrate source used in the fermentation experiments was the fructan Synergy1, a commercially available 1:1 mixture of inulin [Femia et al., 2002] and oligofructose and was obtained from ORAFTI, Tienen, Belgium.

A synthetic fermentation mixture (SFM) mimicking Synergy1 fermentation supernatant (SFS) in the amount of SCFA and DCA (desoxycholic acid) was prepared by mixing required concentrations of SCFA (Na-acetate, Na-butyrate from Merck,

Darmstadt; Germany, Na-propionate from Fisher Scientific, Schwerte, Germany) and DCA (Sigma-Aldrich; Steinheim; Germany) to identify to which proportion the SCFA and DCA might be responsible for the apoptosis inducing activities of the SFS. Additionally, to identify whether bile acids could modify the apoptosis inducing effects of SCFA we investigated SCFA and DCA individually. All synthetic mixtures were prepared by dissolving in respective cell culture medium before use in experiments.

3.1.3 Solutions, reagents and kits

All the solutions, reagents and primers used in the present study are listed in Appendix A and B.

3.1.4 Equipments and instruments

All the equipment and instruments used in the present study are listed in Appendix C.

3.1.5 Softwares

All calculations, statistical analyses and diagrams were made with Graph pad Prism Version 4.0 (Graph Pad, USA) or MS Excel 2002 (Microsoft, USA). The list of softwares is mentioned in Appendix D

3.2 Methods

3.2.1 *In vitro* fermentation of Synergy1

The *in vitro* fermentation of Synergy1 was conducted under anaerobic conditions (80 % Nitrogen, 10 % Carbon dioxide, and 10 % Hydrogen at 37°C) in a batch-culture system with faecal inocula of three different adult healthy donors according to a described procedure [Barry et al., 1995]. All the donors consumed their normal diet

without any restrictions. The use of nutritional supplements and antibiotics were forbidden for the last six months. The samples were weighed and filled in a homogenizing bag. Potassium phosphate buffer (0.1 M, pH 7.0) was added (5:1 v/w) and the mixture was homogenized thoroughly in a Stomacher 400 (Seward U.K). From the faecal homogenate 40 ml aliquots were filled into 500 ml glass bottles. Synergy1 samples were solubilised with anaerobic potassium phosphate buffer to provide 20 g/l fermentable substances (fiber, carbohydrate and protein). 40ml of Synergy1 solution was added to bottles to obtain a final fibre content of 10 g/l and a faecal suspension of 10 % as recommended [Barry et al., 1995]. Each tube was shaken to mix the contents. Anaerobic conditions in the glass bottles were achieved by removing the air with an injected canula and subsequently filled with fermentation gas mixture and incubated at 37 °C for 24 h in a shaking water bath. After placing the suspensions on ice to stop the fermentation, the samples were centrifuged at 6000 g at 4 °C for 30 min to obtain the SFS.

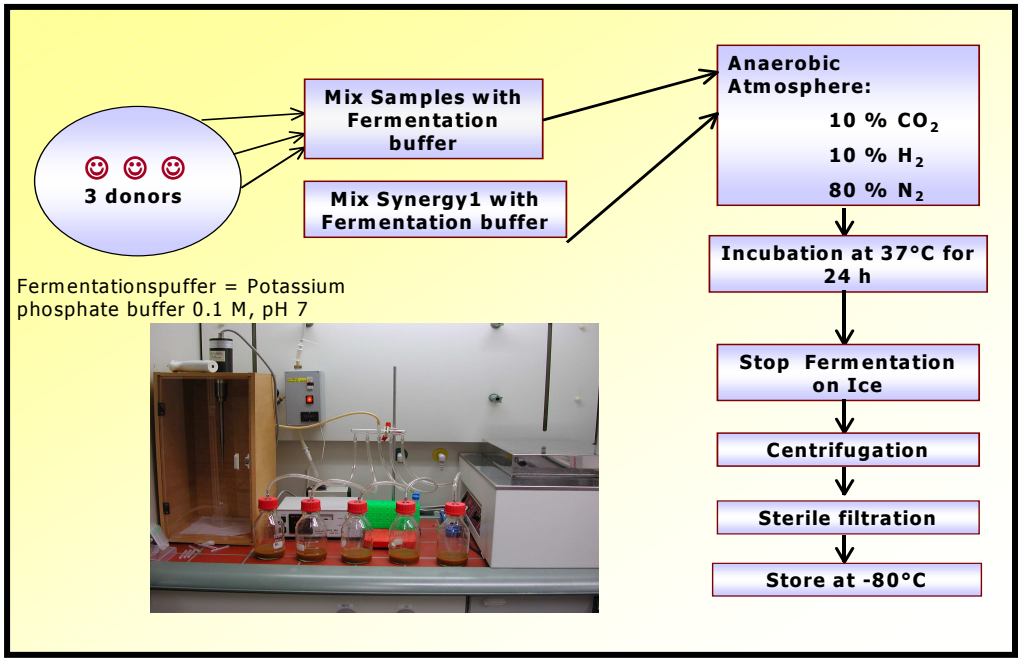


Figure 6 Preparation of Synergy fermentation supernatant (SFS)

A negative control containing only the faecal samples was prepared as faecal blank (FB) (figure 6). The SFS and FB were divided into aliquots and stored at -80°C. Samples were sterilised two times by filtration (pore size 0.45 µm and 0.22 µm) before use.

3.2.2 Analysis of Short chain fatty acids and Desoxycholic acid

Concentrations of SCFA, namely acetate, propionate and butyrate and secondary bile acid DCA were determined by gas chromatography in Department of Nutritional Physiology [Keller and Jahreis, 2004]. Briefly, the samples were mixed with an isocapronate standard (1:11 v/v), shaken, and centrifuged at 6400 g for 10 min. at 4 °C. Thereafter, the gas chromatographic measurements (GC 17A, Shimadzu, Duisburg, Germany) were performed using a 15 m FFAP column (Phenomenex, Aschaffenburg, Germany) and a specific temperature program (start temperature 130 °C; increase 35°C/minute; and a final temperature of 170 °C) [Glei et al., 2006].

To analyse faecal bile acids, aliquots of the samples were hydrolyzed under alkaline conditions with ethanolic NaOH (10 M) at 120 °C for 2 h. The samples were then acidified to pH 1.0 with HCl and extracted with cyclohexane in a tube containing internal bile acid standard (hyodeoxycholic acid). The solvent of the combined extracts were evaporated, and the residues were methylated and silylated. After evaporation, the residual content was resolved in decane, shaken, and centrifuged. The clear solution was injected into a gas chromatograph-mass spectrometer (GC 17-QP 5000, Schimadzu).

3.2.3 Cell culture

Cultivation of cells

Both HT29 and LT97 cells were maintained as adhesion cultures in cell culture flasks (75 cm^2 and 25 cm^2, respectively). For HT29 cells DMEM (Dulbecco's Modified eagle Medium, Invitrogen, Germany) supplemented with 10 % fetal calf serum (FCS) was used. Subconfluent cells were subcultured twice per week by trypsinisation. For this, the old medium was fully removed with a Pasteur pipette and the cells were incubated with 1:10 v/v Trypsin / Versene (5 ml for 75 cm^2 and 2.5 ml for 25 cm^2) for 4 min. Thereafter, Trypsin / Versene was removed and cells were resuspended properly (to avoid cluster formation) in 5 ml of pre-warmed medium. For routine passaging cells were subcultivated at a dilution of 1:5 to 1:10 in cell culture flasks with DMEM supplemented with 10 % FCS.

LT97 cells were maintained in a LT97 culture medium (MCDB 302 containing 20 % L15 Leibovitz medium, 2 % FCS, 0.2 nM Triiodo-L-thyronine, 1 µg / ml Hydrocortisone supplemented with 10 µg / ml Insulin, 2 µg / ml Transferrin, 5 nM Sodium selenite, 30 ng / ml Epidermal growth factor and 50 µg / ml Gentamicin. The medium was changed at regular intervals of 2-3 days (5 ml for 25 cm^2 and 15 ml for 75 cm^2). Therefore, the old medium was fully removed and replaced with pre-warmed medium. The cells were harvested with PBS+Na_2EDTA. For this the cells were first rinsed with PBS+Na_2EDTA followed by incubation with 6 ml PBS+Na_2EDTA for 6-8 min. The PBS+Na_2EDTA solution was then completely removed and the cells were resuspended in 5 ml of pre warmed medium. LT97 cells were subcultured in clusters as they require cell to cell contact for growth. The cells were cultivated in humidified incubator (95 % O_2 and 5 % CO_2) at 37 °C.

Cell counting and determination of vitality

For each experiment the cell number and vitality was measured using CASY TT (Schärfe System GmbH, Germany) cell counter. The principle of this electronic cell counter is based on the measurement of electrical resistance. For this, the cells were

suspended in an isotonic electrolyte solution which is then aspirated by a capillary to which an electric current is applied and the resistance is measured. The increase of resistance is proportional to cell volume. Cell viability can be assessed based on the integrity of plasma membrane as the living cells have intact plasma membranes whereas membranes of dead cells are broken. Therefore, in dead cells only the nuclei account for increase in resistance and vital or dead cells respectively can thus be differentiated. To count cells and determine viability 20 µl of cell suspension were mixed with 10 ml of isotonic CASYton solution which in turn was aspirated by the capillary. The relative cell number is measured as $1x10^6$ cells per ml, whereas the vitality is measured in percent. The absolute cell number was calculated by multiplying relative cell number with the total volume of the cell suspension.

3.2.4 Determination of metabolic activity

To determine metabolic activity in HT29 cells; 8000 cells per well were seeded in 96 well plates. LT 97 cells were however seeded in clusters corresponding to 30-40 % confluency. HT29 cells were incubated with SFS, FB and SFM after 24 h of seeding, whereas LT97 cells were incubated after 5-6 days (according to confluency) in a concentration range of 1.25-20 % v/v. The cells were incubated for 24, 48, and 72 h in a humidified incubator. Metabolic activity (measure of cell viability) was determined by Cell titer blue assay [Klenow et al., 2008]. Two hours before the end of the incubation time 20 µl of cell titer blue (CTB) reagent (Promega, Mannheim, Germany) was added to each well. Metabolic activity was determined by fluorimetrical analysis with Ex/Em 520/595 nm (Spectrafluor Plus, Tecan Germany GmbH, Crailsheim). Results were normalised with cell number determined on the basis of cell growth.

3.2.5 Cell growth assay (DAPI Assay)

The DAPI assay measures effects on growth of cells [Glei et al., 2006] which is based on the amount of DNA present after a definite incubation time. Therefore, this assay measures the relative amount of cells at the end of the incubation. Results were calculated on the basis of medium control set to 100 %.

Effects on cell growth were also determined in 96-well microtiter plates after removal of CTB reagent followed by fixing and permeabilising the cells with 100 µl of methanol for 5 min, and addition of 100µl of 20 µM fluorescent DNA stain, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Germany). After 30 min, DNA content, as a reflection of the number of remaining cells, was detected by fluorimetrical analysis with Ex/Em 360/465 nm in a microplate reader (Spectra Fluor Plus, Tecan, Austria). Results were calculated on the basis of medium control set to 100 %. Mean values (3 determinations per experiment, 3 experiments) were recorded for final evaluation.

3.2.6 Single cell gel electrophoresis assay (Comet assay)

The comet assay has a unique potential for monitoring genetic damage [Tice et al., 2000]. It is the only technique to monitor DNA damage and repair at the level of single cells [Knoll et al., 2005]. The principle behind this assay is that the negatively-charged broken DNA molecules are free to migrate in an electric field towards the anode, with the shorter fragments moving faster (figure 7). The pattern of migration produces a profile resembling the shape of a comet.

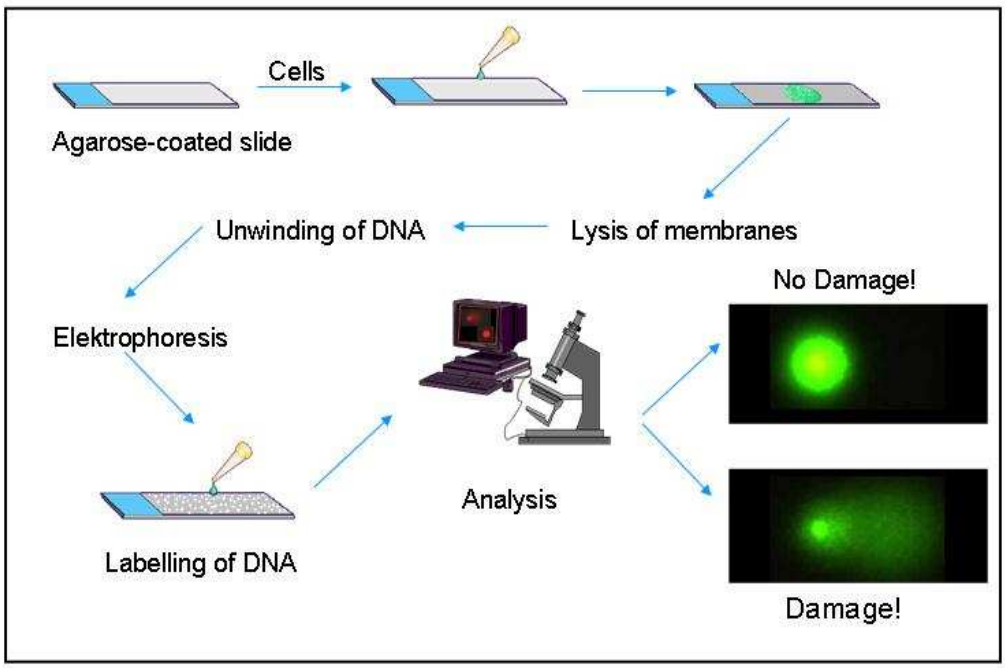


Figure 7 Important steps for performing Comet assay

For long term incubations $2x10^6$ cells HT29 cells were seeded in 6 well plates. The LT97 cells were however, seeded in clusters. The medium was replaced with medium containing 5 % of SFS or FB after 24h of seeding in HT29 cells whereas in LT97 cells the medium was replaced after reaching 50-70 % confluency. The cells were then incubated for 24 h at 37°C in a humidified incubator (5 % CO_2 / 95 % O_2). At the end of incubation the cells were trypsinised and centrifuged (380 x g, 5 min) and washed with PBS. The cell number and vitality was measured using the CASY TT cell counter. The pretreated cells were subsequently incubated with either 75 µM H_2O_2 (diluted in PBS) for 5 min at 4 °C or 300 µM 4-hydroxy-nonenal (HNE) (64 mM stock solution of HNE in 95 % ethanol was diluted with respective media) for 30 min at 37 °C under shaking conditions (380 x g). H_2O_2 and HNE were used as potential model genotoxic substances. H_2O_2 was used as a model substance for free radical intermediates of oxidative stress [Raschke et al., 2006] whereas, HNE is the main lipid

peroxidation product [Knoll et al., 2005]. After incubation genotoxic substances were removed by centrifugation and washing the cells with PBS and subsequently cell viabilities were determined using CASY TT cell counter. The remaining cells were mixed with low-melting agarose and distributed on frosted microscopical slides precoated with normal-melting agarose followed by distribution one more layer of agarose. Further steps were carried out as described elsewhere [Glei et al., 2006]. In short the cells were lysed for 60 min at 4 °C and subjected to alkaline conditions using electrophoresis buffer for 20 min. The electrophoresis was carried out at 25 V and 300 mA and subsequently the slides were neutralized using PBS and stained with SyBr Green (Sigma- Aldrich GmbH, Steinheim, Germany). The slides were analyzed with a fluorescence microscope equipped with a CCD camera as well as a software from Perceptive Instruments namely Comet assay 2.0 (Halstead, United Kingdom). From each slide 150 cells were analyzed and mean values of tail intensities (the percentage of fluorescence in the comet tail) were determined. At least three independently reproduced experiments were performed for statistical analyses.

3.2.7 Enzyme activity assays

Cell seeding and incubation

To determine enzyme activity the cells were seeded in 6 well plates as described above for Comet assay. After 24 h the medium was replaced with 5 % SFS and FB and the cells were incubated for 24 h at 37°C.

Cytosol preparation

The cells were harvested by trypsinisation and cytosols were prepared by resuspending the cells in cold homogenization buffer (250 mM sucrose, 20 mM Tris HCl, 1 mM DTT, and 1 mM Pefabloc; pH 7.4) and homogenized for 1 min using

ultrasound (Bandelin Electronics, Berlin, Germany). Following centrifugation (16000 x g, 60 min, 4°C), the supernatant was aliquoted and stored at -80°C until use.

Glutathione S Transferase (GST activity)

The GST activity was measured spectrophotometrically at 340 nm using 1 mM 1-chloro-2·4-dinitrobenzene (CDNB) and 1 mM glutathione as substrates [Habig et al., 1974]. Therefore 1 mM of glutathione and CDNB were added to a quartz cuvette in a final volume of 1 ml potassium phosphate buffer (pH 6.5; 100 mM). The cuvette was placed in spectrophotometer and the reaction was initiated by addition of cytosol. The increase in absorbance at 340 nm over a 4 min period was measured at 30°C. An extinction coefficient of 9.6 cm/mM was used to determine activity from the initial slope of the reaction. The activity was calculated by 'Lambert-Beer' law (equation 1). The values were expressed as nmol / 10^6 cells / min since this was considered to be more accurate than nmol / µg protein, because environmental factors can enhance general protein content in parallel to induction of GSTs [Treptow-van et al., 1999] and thereby falsify the results

Equation 1 Calculation of total GST enzyme activity

$$\text{Aktivity (nmol} \times \text{min}^{-1} \times \text{ml}^{-1}) = \frac{\Delta E / \text{min} * V}{\varepsilon * d * v} * 1000$$

delta E = change in Extinktion	slope (Abs/min)
V = total volume in cuvette	1 ml
ε = molar Extinktions coefficient	9.6 mM^{-1} cm^{-1} (Habig et al.)
d = thickness of cuvette	1 cm
v = cytosol volume	50-100 µl

Catalase activity

Catalase activity was measured by the method of Aebi [Aebi, 1984]. The principle of this method is based on the hydrolyzation of H_2O_2 and decreasing absorbance at 240 nm since water and oxygen do not absorb at this wavelength. The assay principle is summarized in figure 8:

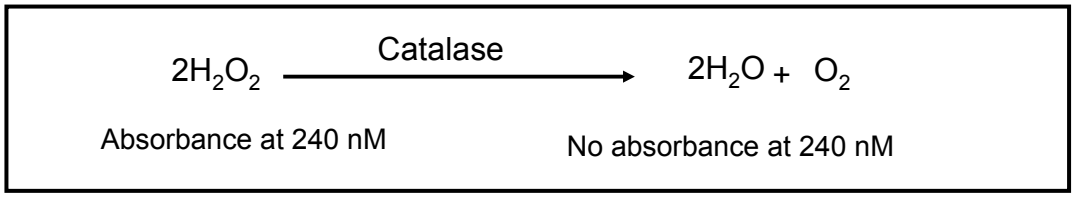


Figure 8 Hydrolysation of H_2O_2 by Catalase

One catalase unit is the amount of enzyme decomposing 1 mM of H_2O_2 per minute at pH 7.0 and 25°C, with an initial H_2O_2 concentration of 10 mM. The activity assay is typically carried out with 10-50 mM H_2O_2 at 25°C.

The assay mixtures used in the experiments contained 10 mM H_2O_2 and 100 µl of cytosols in 50 mM potassium phosphate buffer, pH 7.0. Enzyme activities were calculated using 0.0394 $mM^{-1} \times cm^{-1}$ as absorption coefficient of H_2O_2 at 240 nm.

3.2.8 Determination of Poly (ADP-ribose) polymerase cleavage by Western blot

To analyse effects of SFS and FB on apoptosis, PARP cleavage [Germain et al., 1999] was examined by Western blot. Therefore 2 x 10^6 cells per well were seeded in 6 well plates. After 24 h the medium was replaced with 10 % of SFS, FB and equal concentrations of corresponding SFM, SCFA, DCA and FB. At the end of incubation the cells were lysed using lysis buffer (described below). The chosen concentration of the test substance for apoptosis experiments had a growth inhibiting effect on the cells and was chosen on the basis of EC_{50} from cell growth studies.

Preparation of Cell lysates

Cell lysates were prepared by using cell lysis buffer (20 mM Tris HCl, pH 8.0, 150 mM NaCl, 10 % Glycerol, 2 mM EDTA, 1 mM DTT, 1 % Nonidet P40, 0.5 mM Pefabloc SC, 1 mM PMSF, 1 µg / ml Pepstatin A, 1 µg / ml Leupeptin, 1 mM Sodium orthovanadate). The lysates were then incubated on ice for 20 min and centrifuged at 16000 x g for further 20 min. The pellet containing the debris was discarded and the cell lysates were transferred to new eppendorf tube and analysed for total protein content using the method of Bradford (see below) with bovine serum albumin (BSA) as standard protein. The cell lysates were stored at -20 °C until use.

Determination of protein concentration by Bradford

The Bradford protein assay is a photometric analytical procedure used to measure the concentration of protein in a solution. It is a colorimetric protein assay based on the absorbance shift in the dye Coomasie brilliant blue G when the previously red reagent changes and stabilizes into coomassie blue by the binding of protein (Bradford, 1976). The trimethylmethan dye reacts with basic and aromatic amino acids and forms a complex, which is stable for 30 min and its absorption can be measured at 592 nm. Bovine serum albumin (BSA) in different defined concentrations (5-80 µg/ml) was used to generate a standard curve and allow calculation of unknown protein concentrations in the samples.

Western Blot

Sodiumdodecylsulfate (SDS) was used for separation of proteins. SDS is an anionic detergent which denatures secondary and non-disulfide-linked tertiary structures of proteins and applies a negative charge to each protein in proportion to its mass so that all the proteins move towards the cathode when a current is applied. The cell lysates

were diluted with loading buffer (125 mM Tris, 2 % SDS, 10 % Glycerol, 6 M Urea, 324 mM DTT, and 0.1 % Bromphenolblue) and denatured for 5 min at 99 °C in a thermomixer. 40 µg of total protein was loaded and subjected to SDS gel electrophoresis (stacking gel 4 % w/v; separating gel 10 % w/v acrylamide) for 34-45 min at a constant voltage of 200 V. After separation, the proteins were then blotted by semi dry blotting to a nitrocellulose membrane (Schleicher & Schuell, Germany) for 35-45 min at 80 mA. Thereafter, unspecific binding sites on the nitrocellulose membrane were blocked with blocking solution (1 % milk powder in TBS for at least 1 h. The membrane was incubated overnight with the polyclonal rabbit-anti-PARP antibody (Cell Signalling Technology, Frankfurt, Germany) diluted 1:1000 in blocking solution. PARP is a 116 kDa nuclear protein which is involved in surveillance and maintenance of genome integrity and functions as a key molecule in repair of DNA single strand breaks [Rottenberg et al., 2008]. PARP cleavage is considered to be one of the classical characteristics of apoptosis. During apoptosis, caspases such as caspase-3 and -7, cleave PARP in two fragments of 89 kDa and a 31 kDa [Tewari et al., 1995]. As a loading control a monoclonal mouse-anti-ß-actin antibody (Sigma-Aldrich, Taufkirchen, Germany) in 1:40,000 dilution in blocking solution was used. Following the incubation with the primary antibodies, the membrane was washed two times with TBST (Tris-buffered saline with Tween 20) and one time with TBS (Tris-buffered saline). The blots were probed with corresponding secondary HRP-goat anti-rabbit or HRP-rabbit anti-mouse antibodies (1:800 dilution) for PARP and ß-actin, respectively for 1 h at room temperature. After rinsing the membrane specific bands were visualized by enhanced chemiluminisence using ECL (enhanced chemiluminiscence) Reagent (Amersham, Buckinghamshire, UK). The enzyme horse radish peroxidase (HRP) converts the luminol contained in the ECL reagent into its oxidized form thereby emitting flashes of light. Luminescence can be visualized using photographic films. The densitometric quantification of band intensities was done

using Quantity One 4.1 Software (Bio Rad Munich, Germany). Data was presented as the means ± SD of three independently reproduced experiments.

3.2.9 Caspase activity assay

Caspases are a group of aspartic acid-specific cysteine proteases which are activated during apoptosis. These unique proteases, which are synthesized as zymogens, are involved in the initiation and execution of apoptosis once activated by proteolytic cleavage. In the present study the activity of caspase-8 and -9 (as markers of extrinsic and intrinsic induced apoptosis, respectively) was measured after incubation with SFS and FB. Additionally, activity of caspase-3 was measured as a general marker of apoptosis induction (figure 9)

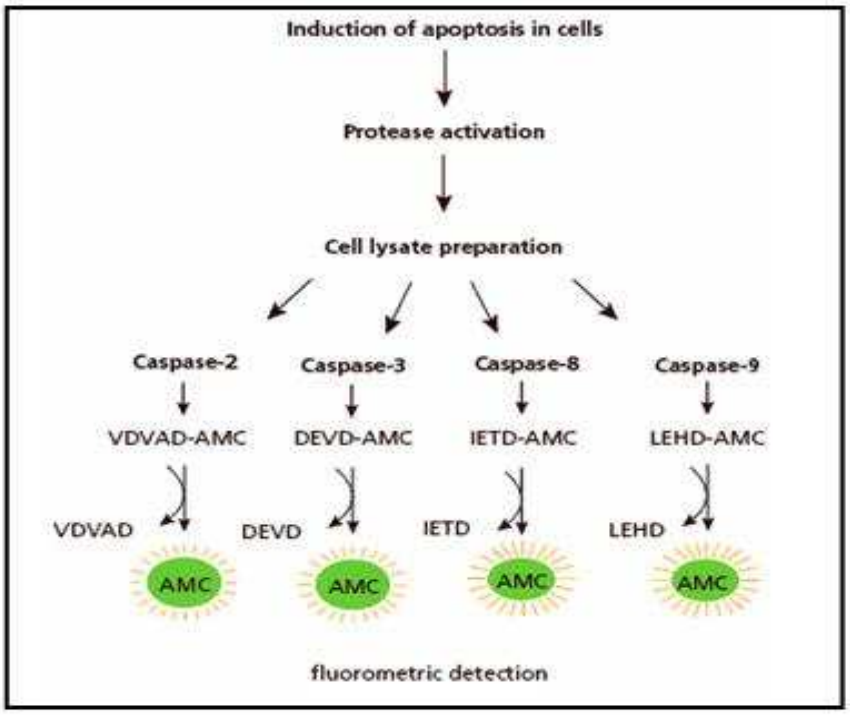


Figure 9 Fluorometric detection of caspase activity during apoptosis (www.clontech.com)

AMC, 7-amino-4- methyl coumarin; Capital letters stand for codes of amino acids

The caspase assay detects the shift in fluorescence of specific substrates. After protease activation, the caspases recognize their respective substrates which are covalently linked to the fluorogenic dye, 7-amino-4- methyl coumarin (AMC). Upon cleavage by the respective caspase, the free dye can be detected using a plate reader (380 nm excitation and 460 nm emission).

To determine the effects on caspase activity cells were seeded in 6 well plates as mentioned above for comet assay. After 24 h the medium was replaced with medium containing 10 % of SFS, FB or SFM respectively. The experiments were performed according to the manufacturer's protocol (ALEXIS Biochemicals, Switzerland). In brief, the cells were harvested after 24 h incubation and cell number and vitality was measured with CASY cell counter. Afterwards the cells were centrifuged at 380 x g for 5 min. The cell pellets were resuspended in chilled lysis buffer (1M HEPES, 100 mM CHAPS, 1 M DTT, 1 mg / ml Leupeptin, 1 mg / ml Pepstatin, 100 mg /ml Pefablock, 100mm Sodium orthovanadate. 100 mM PMSF in Methanol). The cell lysates were kept on ice for 20 min followed by centrifugation at 16000 x g for 15 min at 4°C. Afterwards 50 µl cell lysates were incubated with 2 µl of 50 nM caspase inhibitor (caspase 9: Ac-LEHD-CHO, caspase 3: Ac-DEVD-CHO, caspase 8: Ac-IETD-CHO) for 10 min on ice in 96 well microtitre plates. This step verifies that the signal detected by the kit is due to the specific caspase activity. Next, 100 µl of 37.5 µM of respective substrates (caspase-9-substrate: Ac-LEHD-AMC, caspase-3-substrate: Ac-DEVD-AMC, caspase-8-substrate: Ac-IETD-AMC) were added. The cells were then incubated for 2 h at 37° C at 5 % CO_2 in an incubator. The fluorescence was measured by fluorimetrical analysis with Ex/Em 380/465 nm in a microplate reader (Spectra Fluor Plus, Tecan, Austria). For calculations the fluorescence value of wells containing the inhibitor was subtracted from the fluorescence values of wells containing the substrate. Mean values (3 determinations per experiment, 3 independent experiments) were recorded for the final evaluation.

3.2.10 Real-time RT-PCR

cDNA-Synthesis

2x 10^6 cells per well were seeded in 6 well plates. After 24 h the medium was replaced with medium containing 10 % of SFS or FB. The cells were harvested, centrifuged (2000 rpm / min, 5 min) and washed with PBS. Afterwards RNA was isolated and quantified. These experiments were performed to verify the results of cDNA microarrays.

RNA isolation

2 x 10^6 cells per well were seeded in 6 well plates. After 24 h the medium was replaced with 10 % of SFS or FB. The cells were harvested, centrifuged (380 x g, 5 min) and washed with PBS. The RNA was isolated with RNeasy Plus Mini Kits (Qiagen) [Sauer et al., 2007a], according to manufacturer's instructions. The RNAeasy Plus procedure integrates patented technology for selective removal of double stranded DNA with well established RNAeasy technology.

In brief, cells were first lysed and homogenised in a highly denaturing guanidine-isothiocyanate containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. The lysates were then passed through a gDNA eliminator spin column. This column, in combination with the optimized high salt buffer, allows efficient removal of genomic DNA. Ethanol was then added to the flow through to provide appropriate binding conditions for RNA. The sample was then applied to an RNeasy spin column, where total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA was then eluted in 30 µl of RNAse free water.

RNA quantification and quality control

The quantity of isolated RNA was measured using NanoDrop (ND-1000) (Peqlab Biotechnology Erlangen, Germany). Typically nucleic acids are quantified by UV absorption using a spectrophotometer. In its simplest form the absorbance is measured at 260 and 280 nm. The concentration of nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration.
RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm was used to assess the RNA purity of RNA preparations. Typically, pure RNA has an A260/A280 of 2.1. A value of 1.8-2.0 was used for further experiments.
The integrity of RNA was measured using Agilent RNA 6000 nano kit (Agilent technologies Waldbronn, Germany) according to manufacturer's instructions. Here, total RNA sample was deposited into a RNA Nano Lab Chip. The RNA integrity number (RIN) algorithm was then determined. The RIN is determined to estimate the integrity of total RNA samples. This algorithm assigns a value of 1 to 10 RIN score, where a level of 10 RNA means completely intact RNA. Values between 8 and 10 are optimum [Fleige and Pfaffl, 2006]. Because interpretation of the electropherogram is automatic and not subject to individual interpretation, universal and unbiased comparison of samples is enabled and repeatability of experiments is improved.

First strand synthesis

First-strand cDNA was synthesized by reverse transcription using 2 µg of total RNA, 100 pmol / µl Oligo $(dT)_{12-18}$ primer (Invitrogen, Germany), 5 x First strand buffer (Invitrogen, Germany), 0.1 M DTT, 10 mM dNTPs, 200 U / µl Superscript II RT (Invitrogen, Germany). The reaction was carried out at a programme 65°C for 5 min, 42°C for 60 min, 70°C for 15 min and 4°C forever using a temperature controlled PCR machine (Biometra). Afterwards 1 µl of RNase H (New England Biolabs, Frankfurt,

Germany) was added and the reaction mixture was incubated at 37°C for 20 min to degrade the RNA.

Control PCR

A control PCR was done to check the success of cDNA synthesis. Therefore, the GAPDH (Glycerinaldehyde-3-phosphate dehydrogenase), a house keeping gene was amplified. The control PCR was done by adding 1 µl of cDNA (1:10 diluted with aqua bidest) in a 24 µl of master mix containing 200 µM dNTPs (Invitrogen. Germany), 5 µl of 5 x green buffer (Invitrogen, Germany) 0.2 µM each of reverse and forward primer, 5 U / µl GoTaq DNA (Promega, Germany). The following conditions for the PCR were used: 94°C initial denaturation for 2 min followed by 20 cycles each of 94°C denaturation for 30 sec, 60°C annealing for 30 sec, 72°C extension for 45 sec and a final extension step of 72°C for 5 min. Afterwards 5 µl PCR products were run on a 2 % agarose gel (120 V for 30 min). Additionally 4 µl of 100 bp DNA-Ladder (Peqlab Biotechnology, Erlangen, Germany) was also loaded. Ethidium bromide was added to the agarose gel to visualize the bands. The bands were detected by BioRad System Program Quantity One™ (Bio-Rad Laboratories, München, Germany).

Real-time qRT-PCR using iQ SYBR Green I

Real time PCR monitors the progress of the PCR as it occurs in "real time" by reading fluorescence intensities after each cycle. It is based on the detection and quantitation of a fluorescent reporter. Intensities are proportional to the amount of PCR product generated. Real Time PCR is a kinetic approach in which we can look at the reaction in the early stages while it is still linear. The relative differences in expression levels of selected apoptosis relevant (Bax, DR4, DR5, and Bid), and biotransformation (GSTA4, Catalase) genes were evaluated by real time PCR using the iCycler IQ ® (Bio-Rad GmbH München, Germany) system. GAPDH was used as a reference

control for normalization of the expression levels of all genes of interest. The primer sequences used to amplify genes of interest are shown in Appendix C.

SyBr Green I technique was used to detect the fluorescence. SyBr Green I is cyanin dye which intercalates with double stranded DNA and fluorescence is enormously increased upon binding to double stranded DNA. During the extension phase more and more SyBr Green I will bind to the PCR product, resulting in an increased fluorescence which is measured after each PCR cycle.

The experiments were done using the iCycler iQ real time PCR (Bio-Rad Techonologies München, Germany) according to available instructions. The cycler contains a sensitive camera that monitors the fluorescence in each well of a 96-well plate at frequent intervals during the PCR reaction. Therefore, the cDNA samples were diluted to 1:2.5 and mixed with iQ SYBR Green Supermix (2 x) and 10 pmol each of respective forward and reverse primers. The temperature profile for the iCycler is shown in table1.

Table 1 Real-time PCR temperature profile

	Initial Denaturiation	Denaturation	Annealing	Extension	Terminal Extension
Temperature	95°C	94°C	56°/60°C	72°C	72°C
Time	2 min	30 s	30 s	40 s	10 min
Cycle Number	1	40			1

For apoptosis relevant genes we used an annealing temperature of 56°C whereas, for biotransformation genes a temperature of 60°C was used.

Calculation of relative gene expression levels

For the gene expression analysis of the selected genes a method from Pfaffl was used to calculate relative gene expression levels [Pfaffl, 2001]. Product specific

amplification was confirmed by melting curve analysis. All experiments were performed in triplicates. The fluorescence threshold value (C_T) was calculated by the iCycler iQ® v.3 optical system software. The relative quantification of the target-mRNA expression was calculated with the comparative $\Delta\Delta$ C_T method ($\Delta\Delta C_T = \Delta C_{T\ control} - \Delta C_{T\ experiment}$). The fold change was calculated according to the efficiency method where it is assumed that the PCR efficiency is 100 % (E=2; fold change=$E_{difference}$) (equation 2).

Equation 2: Calculation of gene expression [Pfaffl, 2001]

$$\text{Ratio} = \frac{(E_{target})^{\Delta CT\ target\ (control-treated)}}{(E_{reference})^{\Delta CT\ reference\ (control-treated)}}$$

The ratio shows how much the expression of a gene in a treated sample differs from that of the control sample. The gene expression of the medium-treated cells was set to 1.

3.2.11 Determination of Bid cleavage

Bid relays an apoptotic signal from the cell surface to mitochondria. However, the precise molecular mechanism for the translocation of the cleaved Bid is still unclear. Therefore, the Western blot analysis of cell lysates from both cell lines incubated with SFS & FB for modulation of this marker protein was done in order to get a overview of the cleavage of this apoptosis marker protein.

The cell lysates were prepared as described above for PARP (3.2.13) and proteins were run by SDS polyacrylamide gel electrophoresis (stacking gel 4 % w/v; separating gel 15 % w/v acrylamide). The proteins were blotted on a nitrocellulose membrane which was blocked with blocking solution (5 % milk powder in TBS) for at least 1 h. The membrane was incubated overnight with the polyclonal rabbit-anti-Bid antibody (Cell signalling technology, Frankfurt, Germany) diluted 1:250 in blocking solution. As a loading control a monoclonal mouse-anti-ß-actin antibody diluted to 1:5000 in blocking solution (Sigma-Aldrich, Taufkirchen, Germany) was used. Bid antibody detects endogenous levels of both the full length (22 kDa) and cleaved large fragments (15 kDa) of human Bid [Mandic et al., 2001].

The blots were probed with corresponding secondary HRP-goat anti-rabbit or HRP-rabbit anti-mouse antibodies (1:800 dilution) for Bid and ß-actin, respectively for 1 h at room temperature. After rinsing the membrane specific bands were visualised by enhanced chemiluminisence.

3.2.12 DNA microarray (Custom array)

It was of interest to analyse which genes involved in mechanisms of carcinogenesis or chemoprevention could be modulated by fermentation samples of Synergy1. This was done using a customised cDNA-array system (Miltenyi Biotech, Bergisch Gladbach, Germany) spotted with 300 genes belonging to nine functional categories 1) phase I metabolism, 2) phase II metabolism, 3) phase III metabolism, 4) phase II gene regulation pathway 5) stress and signal transduction pathway, 6) apoptosis signaling pathway 7) tumour suppressor genes, 8) cell cycle arrest / regulation of cell cycle and 9) miscellaneous.

Custom array procedure (PIQOR Microarray, antisense)

A microarray experiment for gene expression profiling consists of RNA preparation and labeling, followed by hybridization of the labeled sample to the microarray. If a limited amount of sample is available, a sample amplification step is included. Here we used PIQOR Microarray, antisense Microarray kit (Miltenyi Biotech, Bergisch Gladbach, Germany) for multiple gene expression. Figure 10 shows the different steps conducted for custom array.

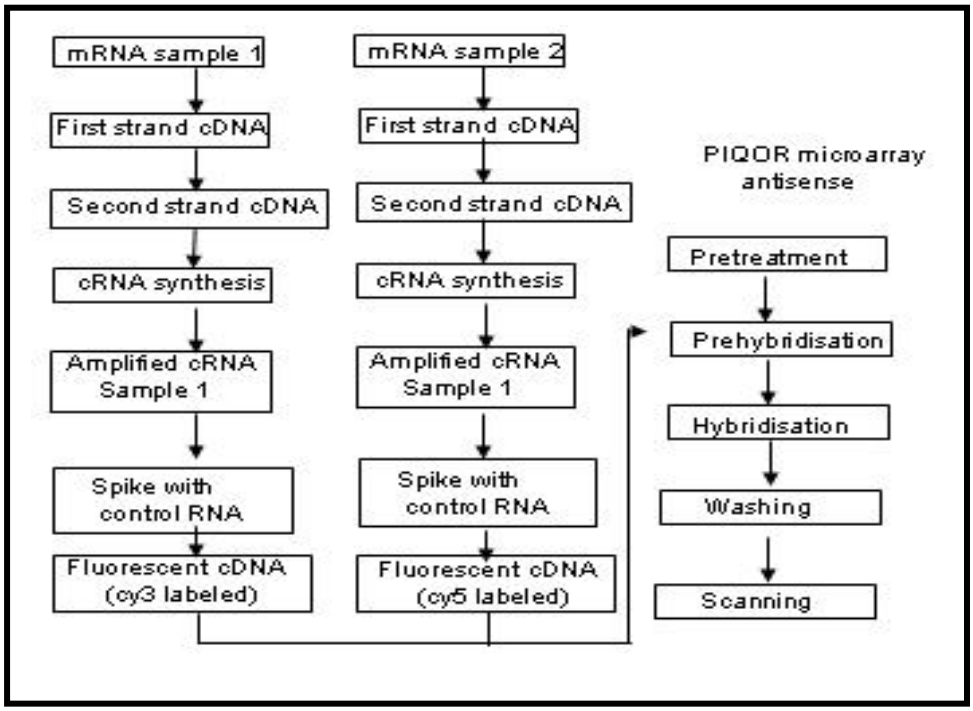


Figure 10 Flowchart for microarray procedure

First and second strand cDNA synthesis

First strand cDNA was synthesized by reverse transcription using 2 µg of total RNA, 100 pmol / µl T7-(T) 24 primer, 5 x First strand buffer, 0.1 M DTT, 10 mM dNTPs, 40 U /µl RNAaseOUT, 200 U / µl Superscript II RT. The reaction was carried out at 70°C

for 10 min, 42°C for 60 min, 70°C for 10 min and 4°C forever using a temperature controlled PCR machine (Biometra, Göttingen, Germany)

Second strand cDNA was then synthesized using RNAse free water, 5x Second strand buffer, 10 U / µl DNA Polymerase I, 10 mM dNTPs, 10 U / µl *E. coli* ligase, 2 U / µl RNase and 20 µl of first strand cDNA and incubating them at 16° C for 2 h using the controlled PCR machine. The cDNA was isolated and purified using Nucleospin® Extract kit, (Macherey & Nagel, Düren, Germany) according to manufacturer's instructions.

cRNA synthesis and clean up

cRNA was then synthesized by *in vitro* transcription (using MEGA script ™ T7 Kit Ambion) according to manufacturer's instructions. In brief 8 µl ds cDNA was added to 75 mM dNTP solution along with T7 RNA polymerase and the mixture was incubated at 37°C for 10 h using controlled PCR machine followed by TURBO DNAase treatment. This DNase treatment removes the template DNA present at a very low concentration relative to RNA. The resulting cRNA was cleaned using NucleoSpin® RNAII kit (Macherey & Nagel, Düren, Germany).

Synthesis of labeled cDNA

The synthesis of fluorescently labeled cDNA was performed using CyScribe GFX, (Amersham Pharmacia) kit according to the manufacturer's instructions. For the labeling reaction 5x first strand buffer, 2 µl Primer mix (PIQOR), 10 mM Low CdNTPs, 25 nmol Cy3-dCTP, 25 nmol Cy5-dCTP and 0.1 M DTT were mixed with 2 µg cRNA and spiked with control RNA 1 and control RNA 2. The reaction was performed using the PCR program PIQOR-cRT (65°C for 5 min cool to 42° C, 60 min) paused at 42°C. 1 µl of SS-RT (200U /µl) was added, mixed and the reaction was

continued at 42°C for 30 min. After 30 min. an additional 1 µl SS-RT was added. After the reaction, remaining cRNA was hydrolysed by addition of 0.5 µl RNase H and incubating the reaction mixture at 37°C for 20 min. The purification of fluorescently labeled cDNA samples was done using CyScribe GFX PCR purification kit (Amersham Pharmacia Biotech, München, Germany) according to manufacturer's instructions.

Hybridisation and analysis of microarray slides

Microarray slides were heated in distilled water using a water bath at 95°C for 2 min followed by treatment with 96 % Ethanol for 30 sec and drying them completely by centrifugation for 3 min at 500 x g. Prehybridisation was done by applying prehybridisation solution to the microarray slides and incubating the slides in humidified hybridisation cassette "PIQOR HybChamb" in a water bath at 65°C for at least 40 min. Afterwards, hybridisation was done by mixing equal amounts of hybridisation solution to cDNA samples and incubating the slides in a humidified hybridisation cassette in a water bath at 65°C overnight. The arrays were then washed with 40 ml wash Buffer I and wash Buffer II (2 times each) for 5 min at 50°C to remove excess of hybridisation solution.

Slides were then sent to Miltenyi Biotech for scanning. Basically, fluorescence signals of the hybridised PIQOR microarrays were detected using a laser scanner from Agilent (Agilent Technologies, Waldbronn, Germany). The mean signal and mean local background intensities were obtained for each spot of the microarray images using the Imagena software (Biodiscovery, California, USA). Low quality spots were flagged and excluded from data analysis. The unflagged spots were analysed with the PIQORTM analyser software (Milltenyi Biotech). This analyser includes background substraction to obtain the net signal intensity, data normalisation and calculation of the Cy5/cy3 ratios. PIQORTM analyser calculates all normalized mean Cy5/Cy3 ratios of the four replicates per gene. Further processing of custom array data was carried

out using Gene Spotter® microarray scanner and Gene spotter v2 6.0 and the mean intensity of all four replicates was calculated. Experiments were reproduced three times and average intensity of each set of probes was used for normalisation based on the GAPDH gene. The differential gene expression was analysed by using the cut off value (≥ 1.5 or ≤0.7 fold change) and changes in genes of interest were confirmed by real time PCR. For defining the significant modulation of gene expression the normalised data of the treatment was compared with that of the control with unpaired t-Tests. The differential expressions of genes were calculated on the basis of significance ($p< 0.05$).

3.2.13 Statistical analysis and graphical presentation

The results were statistically analysed using Sofware GraphPad Prism Version 4.0 (San Diego USA). Means and standard deviations were calculated from at least three independently reproduced experiments. For all the experiments the respective parametric tests were done. Differences between the dataset for proliferation studies were calculated by two way ANOVA and Bonferroni's post hoc test. Here two different groups (control and treatment) were compared with each other on the basis of different concentrations. Other data sets were compared with one way ANOVA and Bonferroni post hoc test. In these cases two groups were tested based on the difference of one factor between them. The Bonferonni post hoc test allows the comparison of each group with a defined group (medium control). For the entire tests a significant difference of $p<0.05$ was taken. For proliferation assays EC_{50} was calculated. EC_{50} is the inhibitory concentration leading to 50 % reduction in cell number and was calculated by non linear regression curve fit. However, custom array results were analysed with two side unpaired t-Test. The significance is shown in respective tables and figures.

4 Results

4.1 Analysis of Short chain fatty acids and secondary bile acids

Dietary fibres escaping enzymatic digestion in the small intestine are delivered to the colon where they are fermented by the anaerobic microflora. It has been shown that dietary fibres modify the intestinal microflora, altering carcinogen metabolism, lower colonic pH and increase faecal SCFA concentration [Pool-Zobel et al., 2005]. Furthermore, they are able to modify faecal bile acid excretion [Owen, 1997]. Therefore, we investigated *in vitro* fermentation samples of Synergy1 for their properties to increase the concentration of SCFA and to decrease toxic secondary bile acids.

Table 2 shows that fermentation of Synergy1 increased the yields of total SCFA in SFS in comparison to FB. In the SFS, the total amount of SCFA was 127.6 mmol/l whereas only 48.1 mmol/l were detected in feces blank (FB) as control. The major products were acetate, propionate and butyrate which were increased after fermentation of Synergy1. Noteworthy, the proportion of butyrate increased four times in SFS compared to FB. The relative molar concentrations, with molar ratios for acetate: propionate: butyrate in FB was 62:22:16 and in SFS was 65:14:21. In addition SFS contained much lower amounts of the potentially toxic secondary bile acid DCA in comparison to FB. No Chenodeoxycholic acid was detectable in the samples.

Table 2 Concentration of SCFA (mmol/l) and DCA (mmol/l) in faeces blank (FB) and Synergy1 fermentation supernatant (SFS)

		FB	SFS
SCFA	Acetate	26.8	80.7
	Propionate	9.2	17.2
	i-Butyrate	1.1	0.6
	n-Butyrate	6.6	26.4
	i-Valerate	1.4	0.8
	n-Valerate	1.8	1.0
	n-Capronate	1.2	0.9
	Total	48.1	127.6
Bile acids	Desoxycholic acid	12.3	3.6
	Chenodeoxycholic acid	-	-

4.2 Effect on cell growth

Cell growth experiments were performed to find out the appropriate subtoxic concentration of test substances (SFS and FB) which can be used for further experiments. Additionally, to investigate, to which proportion SCFA and DCA contribute to the growth inhibitory properties of SFS, we compared these results with a synthetic fermentation mixture (SFM) mimicking the SFS in the amount of SCFA and DCA. The effects of the SFS and FB on growth of LT97 and HT29 cells are shown in Figure 11 and 12. The growth of both cell lines was efficiently reduced in a concentration and time dependant manner. Calculated EC_{50} values (inhibitory concentrations leading to 50 % reduction in cell number) after 48 h and 72 h incubation with SFS were 4.9 % and 4.3 % for LT97 and 10.4 % and 8.7 % for HT29 cells (Table 3). However, no EC_{50} was detectable after 24 h in both cell lines. Anyhow, in LT97 cells a significant decrease in cell growth was already observed after 24 h of treatment (Figure 11).

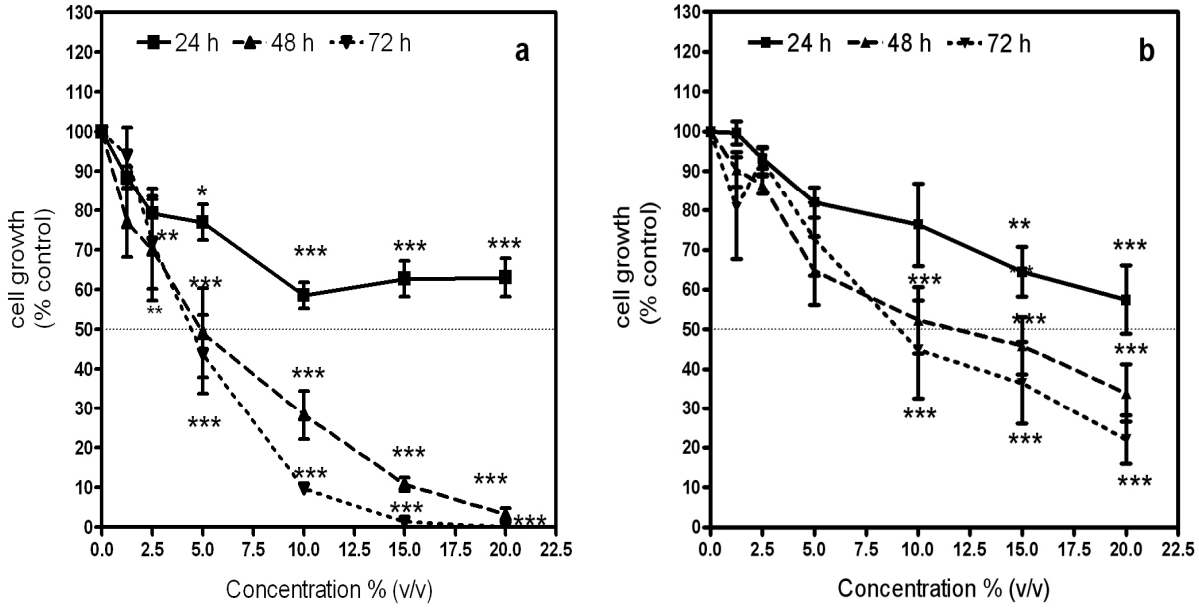


Figure 11 Effect of Synergy1 fermentation supernatant (SFS) (a) and fermentation blank (FB) (b) on growth of LT97 cells

Displayed are means ± SEM (n=4). Statistical variance was analysed with two-way ANOVA and Bonferroni's post hoc test of each concentration compared to medium control (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

The growth inhibition in HT29 cells was observed later, reaching significance at ≥ 10 % SFS after 48 h of incubation (Figure 12). The treatment of cells with FB also inhibited growth of both cell lines, but the growth inhibiting effect was weaker and not comparable to SFS in the above mentioned manner and EC_{50} could only be observed after 72 h of incubation with FB (Table 2). Additionally, incubation of HT29 cells with low concentrations (2.5 and 10 %) of SFS and FB resulted in moderate increase in cell growth (>100 %).

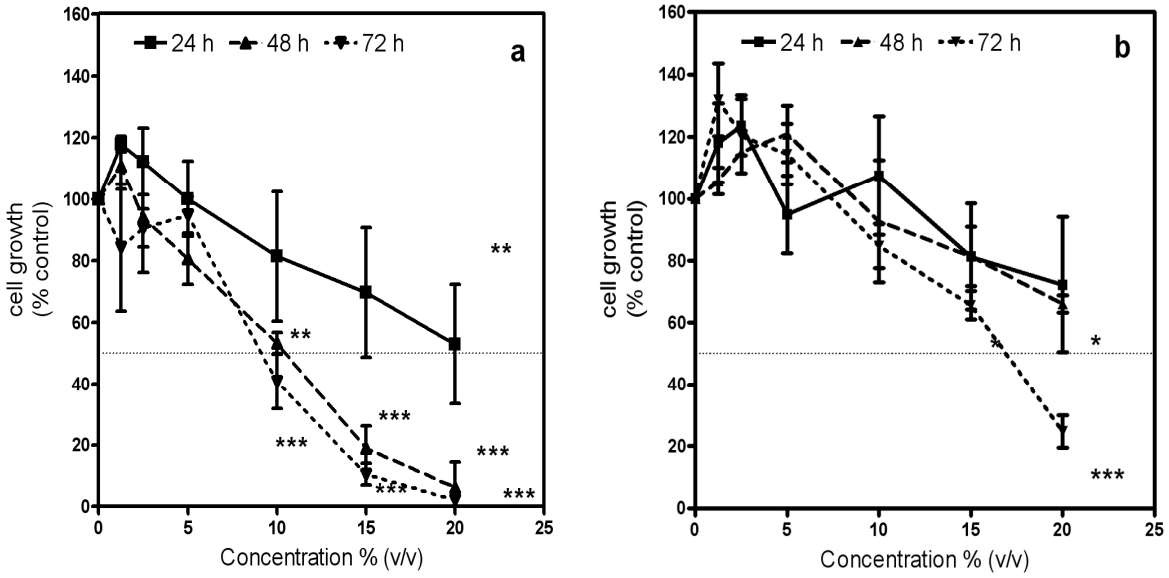


Figure 12 Effect of Synergy 1 fermentation supernatant (SFS) (a) and fermentation blank (FB) (b) on growth of HT29 cells.

Displayed are means ± SEM (n=4). Statistical variance was analysed with two-way ANOVA and Bonferroni's post hoc test of each concentration compared to medium control (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Comparison of the EC_{50} values for both cell lines shown in Table 3 indicates that growth inhibitory effects of SFS and SFM were much stronger in LT97 cells than in HT29 cells. The SFM however found to be almost equally effective as SFS in inhibiting the growth in both cell lines when EC_{50} was compared.

Table 3 EC_{50} (inhibitory concentrations leading to 50 % reduction of cell number) after treatment of LT97 and HT29 cells for 24-72h with Synergy1 fermentation supernatant (SFS), Synthetic fermentation mixture (SFM) and faeces blank (FB)

Time of Incubation	SFS		SFM		FB	
	LT97	HT29	LT97	HT29	LT97	HT29
24 h	-	-	-	-	-	-
48 h	4.9 %	10.4 %	3.2 %	12.0 %	10.7 %	-
72 h	4.3 %	8.7 %	3.1 %	7.3 %	10.0 %	17.6 %

4.3 Effect on metabolic activity

Figure 13 compares the effects of SFS (1.25-20 %) on metabolic activity of both cell lines incubated for 24-72 h. The results show that metabolic activity of the remaining cells (HT29 & LT97) was not significantly affected by incubation up to 48 h with SFS. Only the incubation for 72 h with 15 and 20 % SFS resulted in a significant increase in metabolic activity in LT97 cells, whereas only the highest tested dose of SFS (20 %) significantly increased the metabolic activity of HT29 cells.

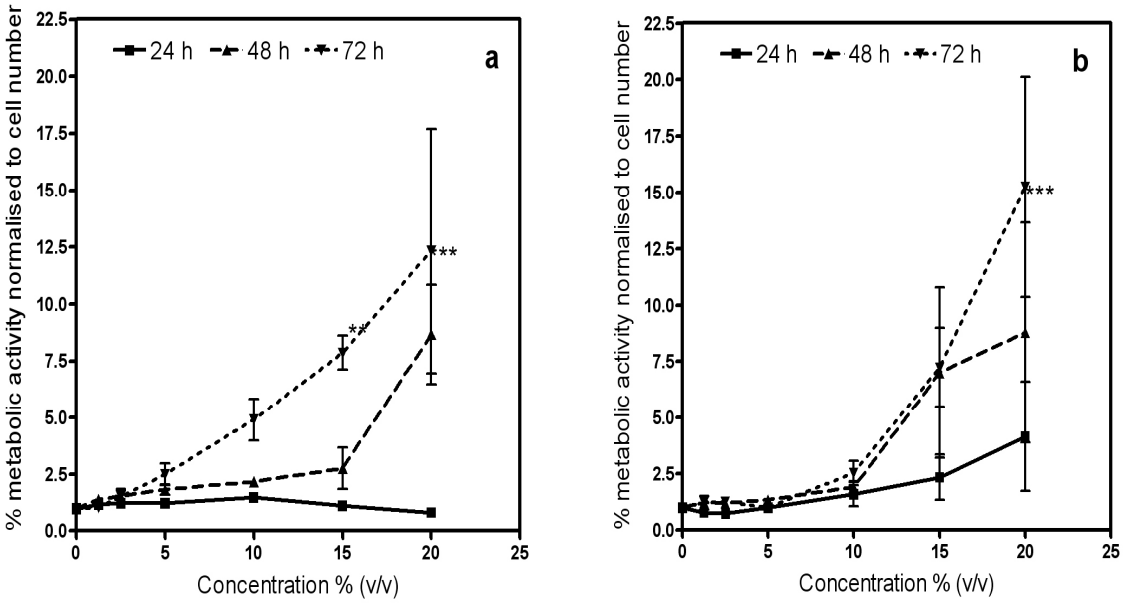


Figure 13 Effect Synergy 1 fermentation supernatant (SFS) on the metabolic activity of LT97 cells (a) and HT29 (b) cells.

Displayed are means ± SEM (n=4). Statistical variance was analysed with two-way ANOVA and Bonferroni's post hoc test of each concentration compared to medium control (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

In contrast, treatment of both the cell lines with FB (20 %) after 72 h resulted in only a minor (3 %) in metabolic activity (data not shown). The results underline that the lower concentration of SFS tested (5 and 10 %) inhibited cell growth but

did not impair the metabolic activity of the remaining viable cells. Therefore, the metabolic status of the remaining cells was not influenced by the concentrations of SFS used in the further experiments.

4.4 Modulation of DNA damage (Comet assay)

The Comet assay detects DNA strand breaks in single cells and was used here in order to test genotoxic / anti-genotoxic activities of fermentation products of Synergy1. This assay does not give any information about the mutagenicity of the test substances, but it provides important information about the possible damage to DNA integrity [Collins, 2004].

4.4.1 Analysis of genotoxic effects of Synergy1 fermentation supernatant

To measure the genotoxic potential of the test compounds, SFS and FB were investigated at a concentration of 5 % for 24 h. In this amount the effective concentrations of acetate, propionate and butyrate in SFS and FB were 16.2 mM, 3.4 mM, 5.2 mM and 5.4 mM, 1.8 mM and 1.3 mM respectively. Additionally, the viability of both the cell lines remained above 80 % after incubation with SFS and FB.

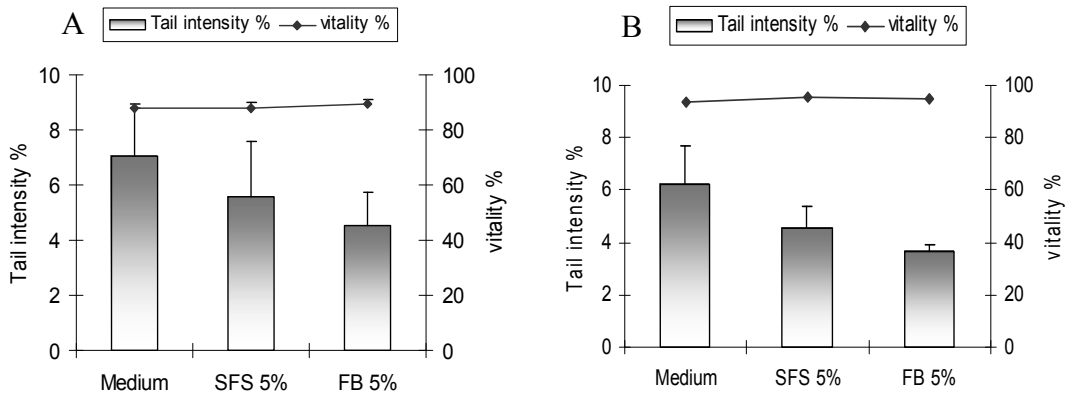


Figure 14 Effect of preincubation with Synergy1 fermentation supernatant (SFS) and fermentation blank (FB) for 24 h on DNA damage of LT97 (A) and HT29 cells (B)

Displayed are the means and SD of 3 independently reproduced experiments One way ANOVA, Bonferroni post-tests (SFS 5 %: Synergy fermentation supernatant 5 %, FB 5 %: Fermentation Blank 5 %.

Figure 14 shows clearly that incubation of LT97 and HT29 cells with SFS and FB did not result in any genotoxic effect. The moderate decrease in tail intensity did not result in significance in both cell lines.

4.4.2 Analysis of anti-genotoxic effects Synergy fermentation supernatant

A challenge assay was done in order to determine the possible protective effects of SFS against induced DNA damage. Therefore, two genotoxic agents namely H_2O_2 and HNE were chosen. Both HT29 and LT97 cells were pre-incubated with 5 % SFS or 5 % FB for 24 h to test the influence on the toxicological defence of the cells. The cells pre-incubated with medium were used as untreated controls. Subsequent to this pre-incubation, the cells were exposed to 75 µM H_2O_2 for 5 min or 300 µM HNE for 30 min. Figure 15 & Figure 16 show that both H_2O_2 and HNE were clearly genotoxic without any indication of concomitant cytotoxicity. Cell viability determined by CASY cell counter was always > 80 %.

Pre-incubation with SFS resulted in significantly reduced levels of H_2O_2-induced DNA strand breaks (39 % in medium control to 22 % in SFS) in HT29 cells ($p \leq 0.01$) whereas this effect was not significant in LT97 cells (figure 15).

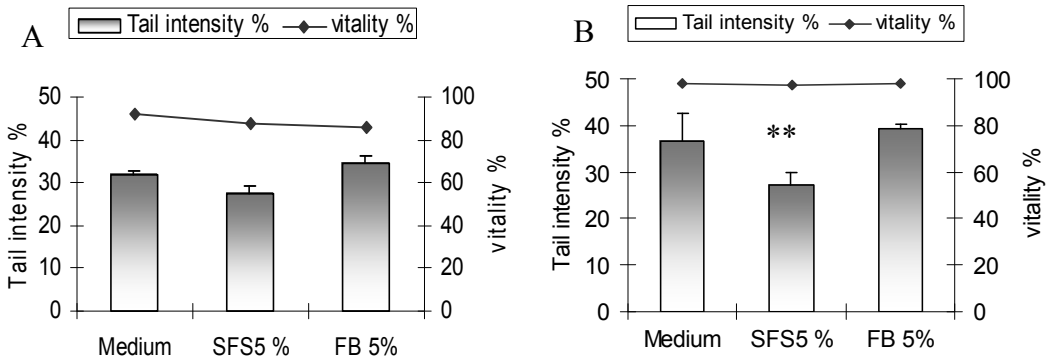


Figure 15 H_2O_2-induced DNA damage (75 µM) after pretreatment of LT97 (A) and HT29 cells (B) with Synergy1 fermentation supernatant (SFS) and fermentation blank (FB) for 24 h.

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests **p<0.01; (SFS 5 % Synergy fermentation supernatant 5 %, FB 5 % Fermentation Blank 5 %.

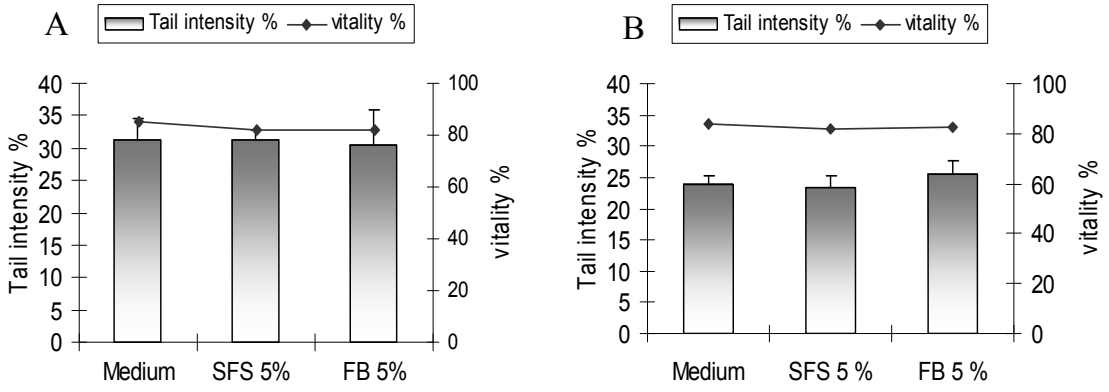


Figure 16 HNE-induced DNA damage (75 µM) after pretreatment of LT97 (A) and HT29 cells (B) with Synergy1 fermentation supernatant (SFS) and fermentation blank (FB) for 24 h.

Displayed are the means and SD of 3 independently reproduced experiments One way ANOVA, Bonferroni post-tests (SFS 5 % Synergy fermentation supernatant 5 %, FB 5 % Fermentation Blank 5 %.

In contrast to the results with H_2O_2, the SFS was unable to modulate the genotoxic effects of HNE (figure. 16).

4.5 Effects on mRNA expression of Glutathione-S-Transferase and Catalase

To investigate the possible mechanisms of detoxification involving modulation of mRNA levels; both cell lines were incubated with 5 % SFS and FB to determine the effects on gene expression of GSTA4 and CAT using real-time RT-PCR. Both GSTA4 and CAT have very high substrate specificity towards genotoxins namely HNE [Knoll et al., 2005] and H_2O_2 [Sauer et al., 2007a] and thus help in detoxification in the body. These genotoxins react with large biomolecules such as DNA and lipids in the body which in turn can lead to mutations. Therefore, the up-regulation of the analysed genes at the mRNA level may be an important step in the prevention of cellular damage by free radicals. Here, both the cell lines were incubated with 5 % SFS and FB for 24 h to determine the effects on gene expression of GSTA4 and CAT using real-time RT-PCR. The effects on gene expression are shown in figure.17 and figure 18. In general, incubation with SFS resulted in a significant up-regulation of GSTA4 (fold change 2.4) in HT29 tumour cells. The expression of CAT however increased ony non-significantly in both cell lines (fold change of 1.85 and 1.33 in HT29 and LT97 cells respectively. Noteworthy, the faeces blank (FB) also tended to significantly increase GSTA4 gene expression in HT29 cells (figure 17).

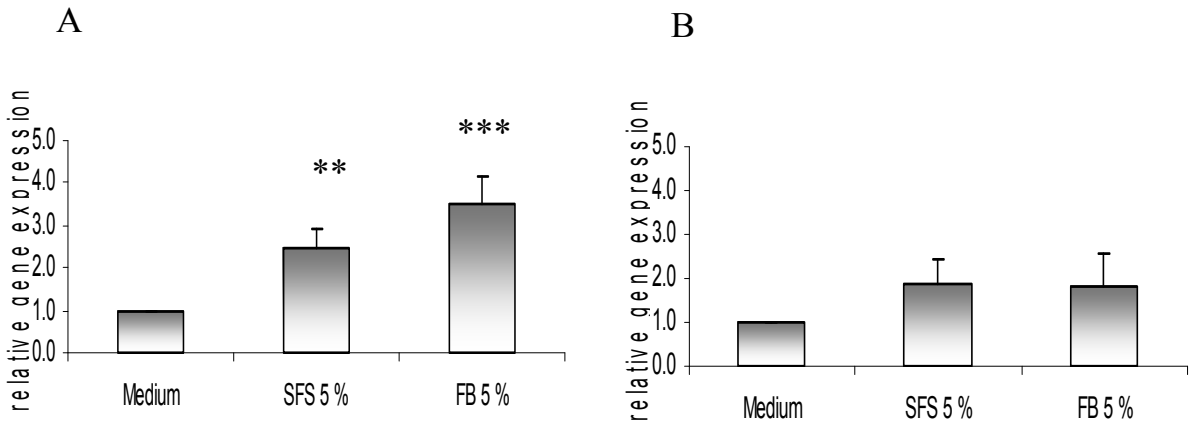


Figure 17 mRNA expression of GSTA4 (A) and CAT (B) in HT29 cells after incubation with Synergy1 fermentation supernatant (SFS) and fermentation blank (FB)

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. **p<0.01;***p<0.001 (SFS 5 % Synergy fermentation supernatant 5 %, FB 5 % Fermentation Blank 5 %)

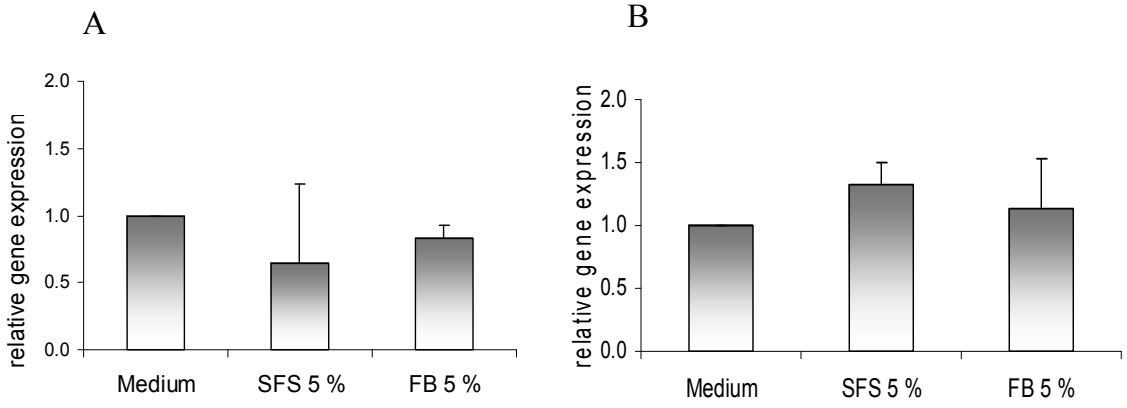


Figure 18 mRNA expression of GSTA4 (A) and CAT (B) genes in LT97 cells after incubation with Synergy1 fermentation supernatant (SFS) and fermentation blank (FB)

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. (SFS 5 % Synergy fermentation supernatant 5 %, FB 5 % Fermentation Blank 5 %)

4.6 Phase II enzyme activity

4.6.1 Effect on Glutathione-S-Transferase activity

One possible mechanism by which SFS could decrease the genotoxicity of potential carcinogens is the enhancement of GST activity. Therefore, the effect of 5 % SFS on total GST enzyme activity was measured. The GSTs constitute one major family of phase II detoxification enzymes involved in the cellular protection against electrophilic intermediates. Although the basal activity (in medium control) of GSTs in LT97 cells was much lower than in HT29 cells, there was a small trend for a increased GST activity resulting from incubation of both cells lines for 24 h (figure 19). However, this increase was not significant. The FB had no impact on GST activity too.

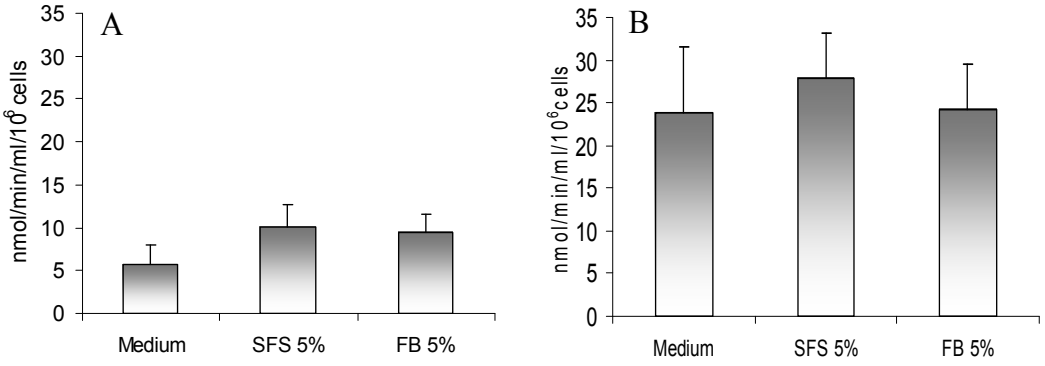


Figure 19 GST activity / 10^6 cells for LT 97 (A) and HT 29 (B) cells after incubation with 5 % Synergy1 fermentation supernatant (SFS) and fermentation blank (FB) for 24 h.

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. (SFS 5 % Synergy fermentation supernatant 5 %; FB 5 % Fermentation Blank 5 %)

4.6.2 Effect on Catalase activity

Another possible mechanism by which SFS could decrease genotoxicity of oxidative stressors could be an enhancement of catalase activity. Catalase is a common enzyme found in nearly all living organisms and is a hydrogenperoxide oxidoreductase [Patlolla et al., 2008]. Its function includes catalysing the decomposition of H_2O_2 to water and oxygen. Compared to medium control and FB, SFS treatment resulted in a significant induction of catalase activity ($p<0.5$) in HT29 cells (figure 20). Although there was an increasing trend of catalase activity in LT97cells but this was not significant. The incubation with FB control however had no effect in both cell lines.

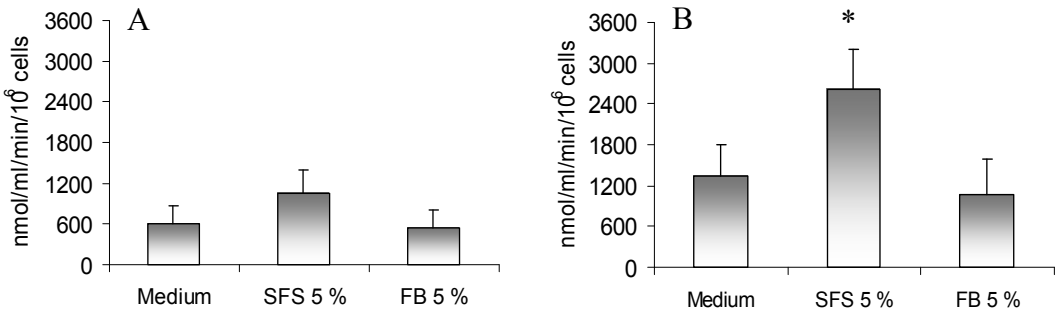


Figure 20 Catalase activity / 10^6 cells for LT 97 (A) and HT 29 (B) cells after incubation with 5 % Synergy1 fermentation supernatant (SFS) and fermentation blank (FB) for 24 h.

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. *$p<0.5$ (SFS 5 % Synergy fermentation supernatant 5 %, FB 5 % Fermentation Blank 5 %)

4.7 Effect on PARP cleavage

Resistance to death by apoptosis is one of the hallmarks of cancer cells [Hanahan and Weinberg, 2000] and this factor, together with maintained or enhanced rates of

cell proliferation, contributes to expansion of the tumour mass [Hague et al., 1993]. Studies show that incubation with butyrate results in tumour cell apoptosis by up-regulating the expression of the pro-apoptotic protein BCL-2 homologous antagonist/killer (BaK) and induces caspase-3 mediated cleavage of target proteins including PARP [Chai et al., 2000]. The latter is cleaved after Asp^{777} to yield a characteristic and stable 89 kDa fragment [Browne et al., 1998]. In order to get general overview about the SFS induced apoptosis, PARP cleavage was measured by incubating the cells with both 5 and 10 % of SFS and FB. The Western blot analysis revealed specific, cleavage of PARP upon incubation of LT97 cells with SFS demonstrating induction of apoptosis. The results in figure 21 show that treatment of LT97 cells for 24 h with SFS significantly increased cleavage of PARP when compared to medium control. However, in HT29 cells, 24 h treatment did not lead to increase of PARP cleavage indicating that HT29 cells were not sensitive towards SFS-induced apoptosis. Additionally, 10 % of FB was also able to induce a significant PARP cleavage in LT97 cells after 24 h of incubation.

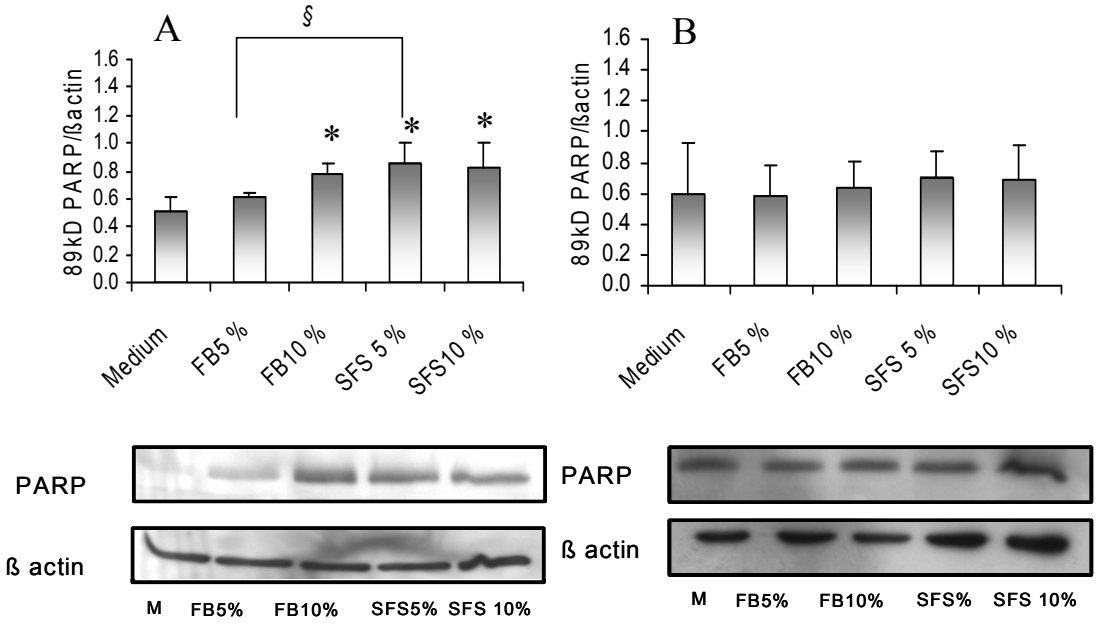


Figure 21 PARP-cleavage induced by Synergy1 fermentation supernatant (SFS) and fermentation blank (FB) in LT97 (A) and HT29 (B) cells after 24 h incubation

Displayed are means ± SD (n=3). Statistical variance was analysed by one-way ANOVA and Bonferroni's post hoc comparison test of each concentration compared to medium control (*p<0.05). Differences between SFS and FB were analysed by two way ANOVA (§p<0.05). (SFS 5 % Synergy fermentation supernatant 5 %, SFS 10 % Synergy fermentation supernatant 10 %, FB 5 % Fermentation Blank 5 %, FB 10 % Fermentation Blank 10 %)

Additionally, in order to find out whether PARP cleavage induced by SFS is due to the modulation of metabolite composition (increase in SCFA and decrease in DCA); both cell lines were incubated with SFM mimicking SFS in the concentration of SCFA and DCA. Incubation of LT97 cells for 24 h (figure 22) with synthetic SCFA mixture resulted in a significant increase in PARP cleavage. However, the levels of cleaved PARP with SFM were lower in comparison to SFS which points to the role of some additional apoptosis inducing substances in complex SFS.

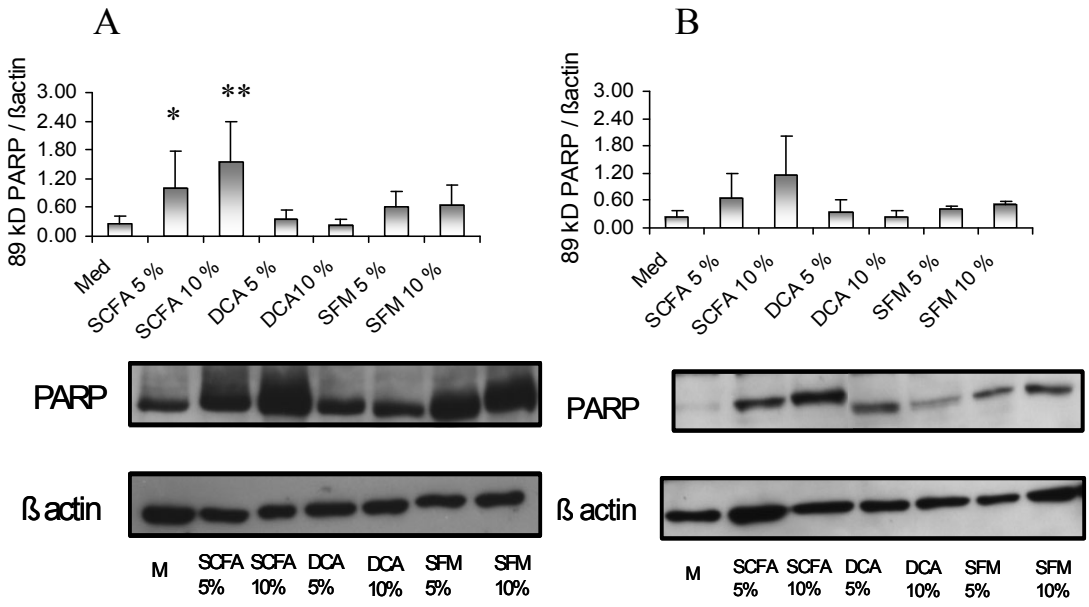


Figure 22 PARP-cleavage induced by Synthetic fermentation mixture (SFM) in LT97 (A) and HT29 (B) cells after 24 h.

Displayed are means ± SD (n=3). Statistical variance was analysed by one-way ANOVA and Bonferroni's post hoc test of each concentration compared to medium control (*p<0.05, **p<0.01). (SCFA5 % Short chain fatty acid mixture 5 %, SCFA 10 % Short chain fatty acid

mixture 10 %, DCA 5 % Desoxycholic acid 5 %, DCA 10 % Desoxycholic acid 10 %, SFM5 % Synthetic fermentation mixture 5 %, SFM10 % Synthetic fermentation mixture 10 %)

4.8 Effect on Caspase activity

In order to get more information about the possible mechanisms of apoptosis induction (extrinsic or intrinsic) we determined the caspase activity. Therefore, in relation to apoptosis we measured the activity of 3 different caspases namely caspase-3, caspase-8 and caspase-9 after incubation of both cell lines with 10 % of SFS and FB for 24 h because this concentration had a significant impact on cell growth. In LT97 cells, in general the activity of all the investigated caspases were increased after incubation with SFS (figure 23).

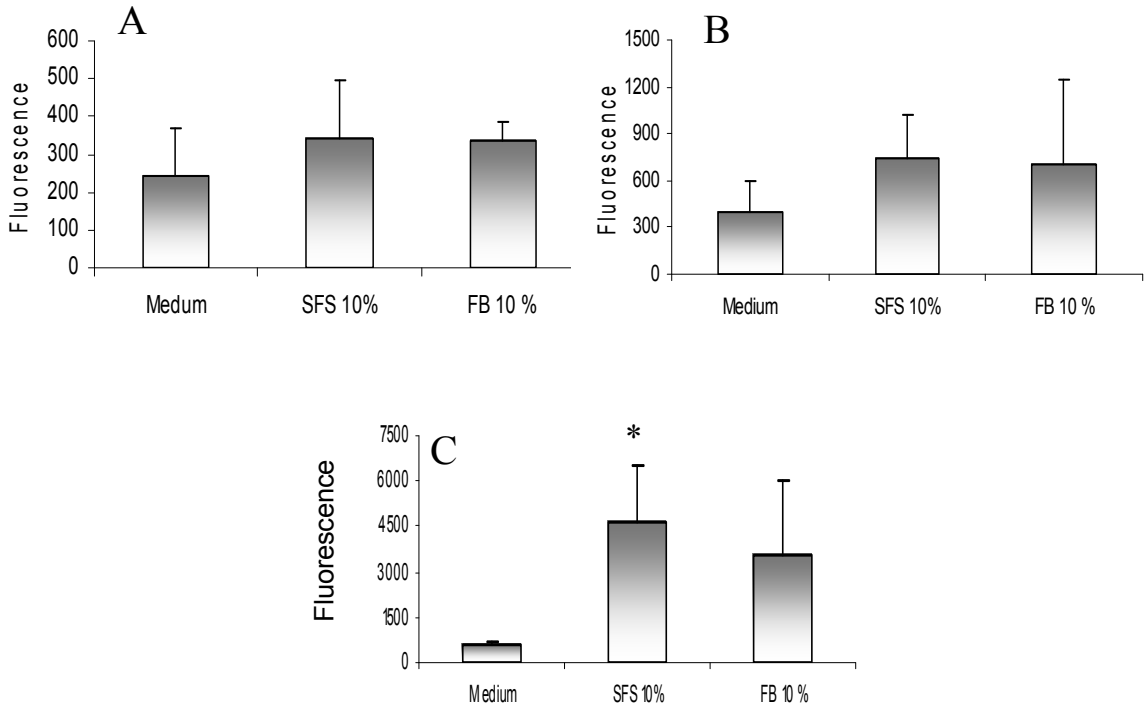


Figure 23 Effect of incubation with Synergy1 fermentation supernatant (SFS 10 %) and fermentation blank (FB 10 %) on Caspase-9 (A) Caspase-8 (B) Caspase-3 (C) in LT97 cells.

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. *p<0.05 (SFS 10 % Synergy fermentation supernatant 10 %, FB 10 %: fermentation Blank 10 %)

However, only the activity of caspase-3 was increased significantly after 24 h of incubation (p<0.05). Among caspase-8 and -9 a trend of increased activity was visible. In addition to SFS, incubation of LT97 cells with FB also resulted in an up regulation of caspase-9,-3 and- 8 but the activity was not significant at all.

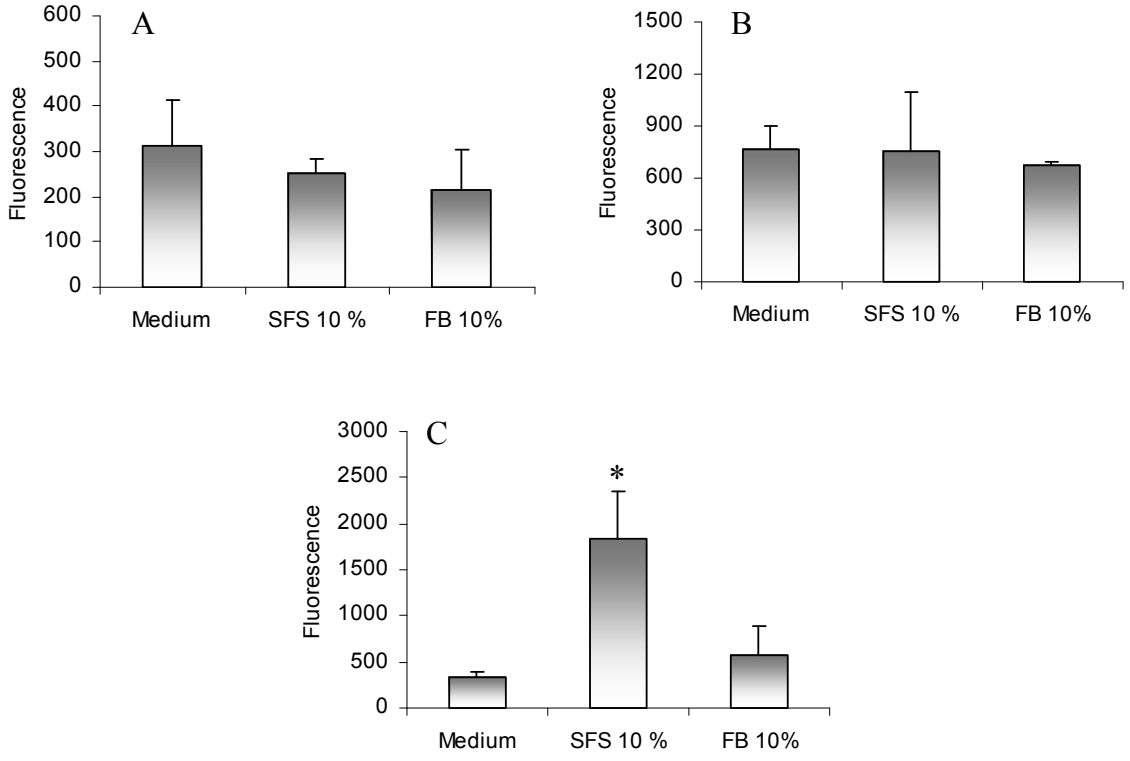


Figure 24 Effect of incubation with Synergy1 fermentation supernatant (SFS 10 %) and fermentation blank (FB 10 %) on Caspase-9 (A), Caspase-8 (B), Caspase-3 (C) in HT29 cells

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. *p<0.05 (SFS 10 % Synergy fermentation supernatant 10 %, FB 10 %: Fermentation Blank 10 %)

In HT29 cells, a significant increase in activity of caspase-3 was visible after incubation with SFS for 24 h. However, there was no change in the activity of caspase-8 and-9 (figure 24). Additionally, there was no change in the activity of all the three investigated caspases in HT29 cells after incubation with FB 10 % for 24 h. Thus, the activity of caspase-3 was most clearly modulated after incubation of both cell lines with SFS pointing towards the role of some common apoptosis inducing mechanisms which converge at caspase-3.

Further, incubation of both cell lines with SFM also lead to a significant increase in the activity of caspase-3 (figure 25) pointing towards the role of change in the concentrations of active metabolites during fermentation procedure.

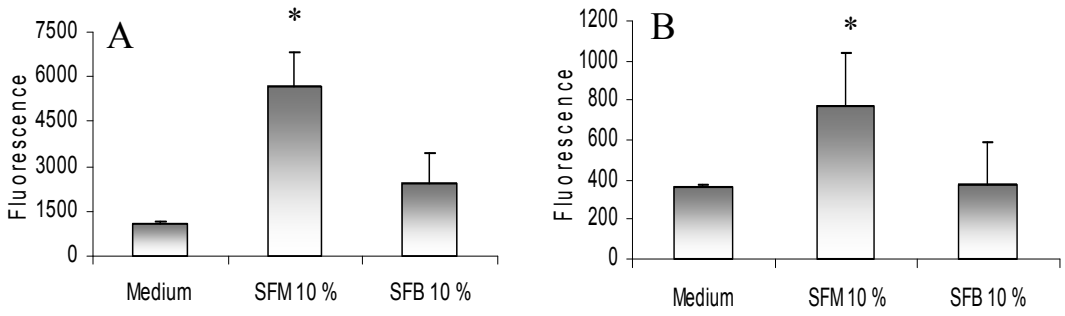


Figure 25 Effect of incubation with SFM on caspase-3 activity in LT97 cells (A) and HT29 (B) cells

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. *$p<0.05$ (SFS 10 % Synergy fermentation supernatant 10 %, FB 10 %: Fermentation Blank 10 %)

4.9 Effect on apoptosis relevant genes

In order to get detailed information on the apoptosis inducing mechanisms of SFS at the mRNA level, both cell lines were incubated with 10 % SFS and FB for 24 h and the modulation in the apoptosis relevant genes namely DR4, DR5, Bid and Bax were investigated. DR4 and DR5 are members of the tumour necrosis factor receptor family [Thorburn, 2004]. Bid is the BH3 domain-only protein and is a death agonist member of the Bcl-2 family [Wang et al., 1996] whereas Bax is a key component for cellular induced apoptosis [Wei et al., 2001]. All these genes play an important role in the regulation of apoptosis.

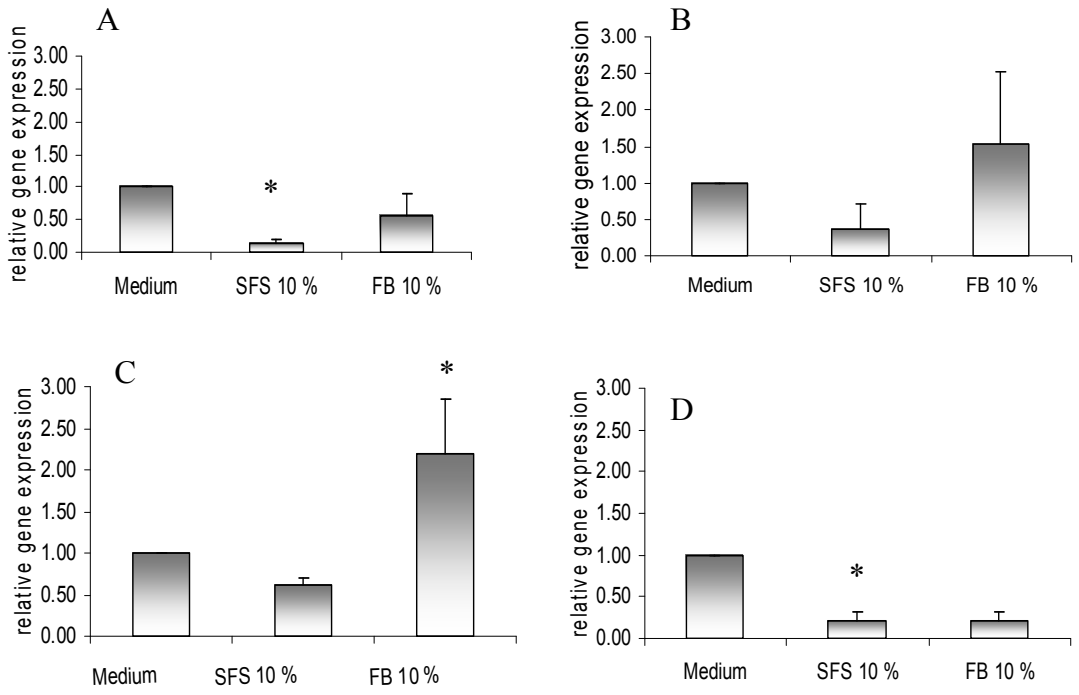


Figure 26 Expression of apoptosis relevant genes DR4 (A), DR5 (B) Bid (C) and Bax (D) genes in LT97 after incubation with Synergy1 fermentation supernatant (SFS 10 %) and fermentation blank (FB 10 %) analysed with real time PCR.

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. *p<0.05 (SFS 10 % Synergy fermentation supernatant 10 %, FB 10 %: Fermentation Blank 10 %)

According to our data analysis of RT-PCR, DR4 and Bax were down-regulated significantly (0.13 fold and 0.2 fold respectively) after incubation of LT97 cells with SFS (figure 26). The incubation of cells with FB resulted in a significant up-regulation of Bid (2.19 fold). In contrast to LT97 cells, we observed a significant up-regulation of mRNA expression of DR5 and Bax (fold change 2.15 and 1.68 respectively) in HT29 cells when compared to medium control (figure 27). There was, however, a significant down-regulation of DR4 (fold change 0.33) after incubation of HT29 cells with SFS10 %.

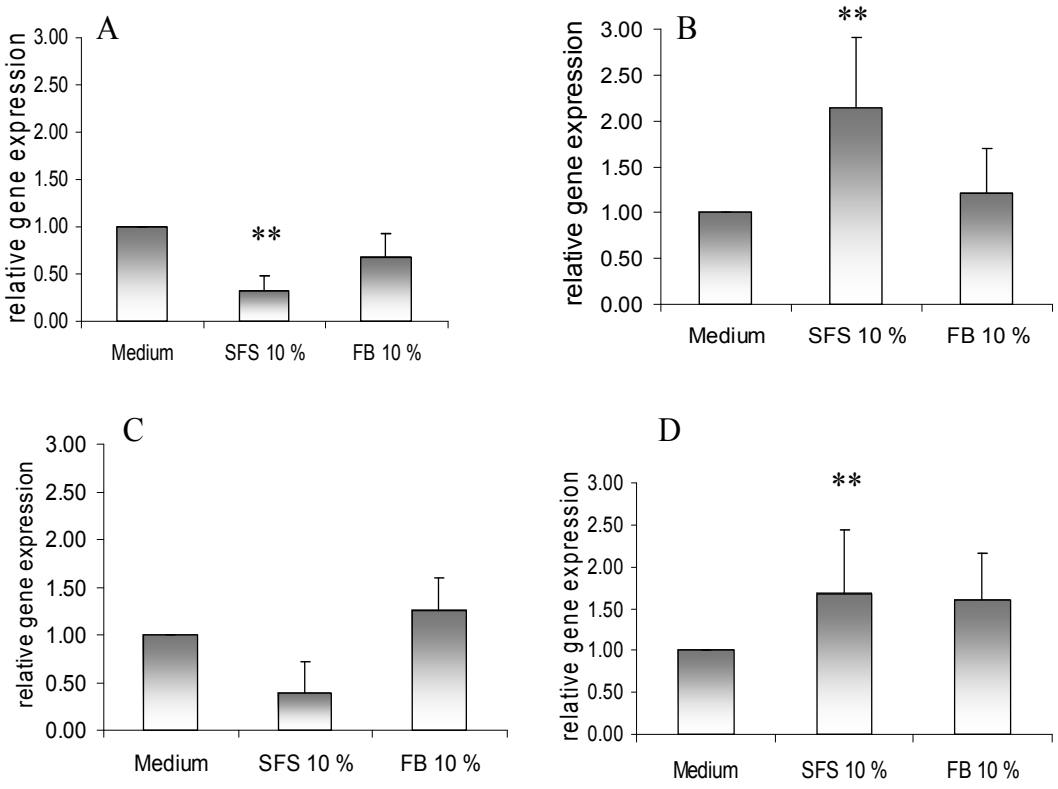


Figure 27 Expression of apoptosis relevant genes DR4 (A), DR5 (B) Bid (C) and Bax (D) genes in HT29 cells after incubation with Synergy1 fermentation supernatant (SFS) and fermentation blank (FB) analysed with real time PCR.

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. **p<0.01 (SFS 10 % Synergy fermentation supernatant 10 %, FB 10 %: Fermentation Blank 10 %)

4.10 Effect on Bid Cleavage

In addition to real time analysis the expression of Bid was also analysed at the protein level by Western blot. Bid antibody (human specific) detects endogenous levels of both the full length (22 kDa) and cleaved fragment (15 kDa) of human Bid. Here, we measured the levels of full length Bid after incubation of both the cell lines with SFS and FB 10 % for 24 h. The results are shown in figure 28.

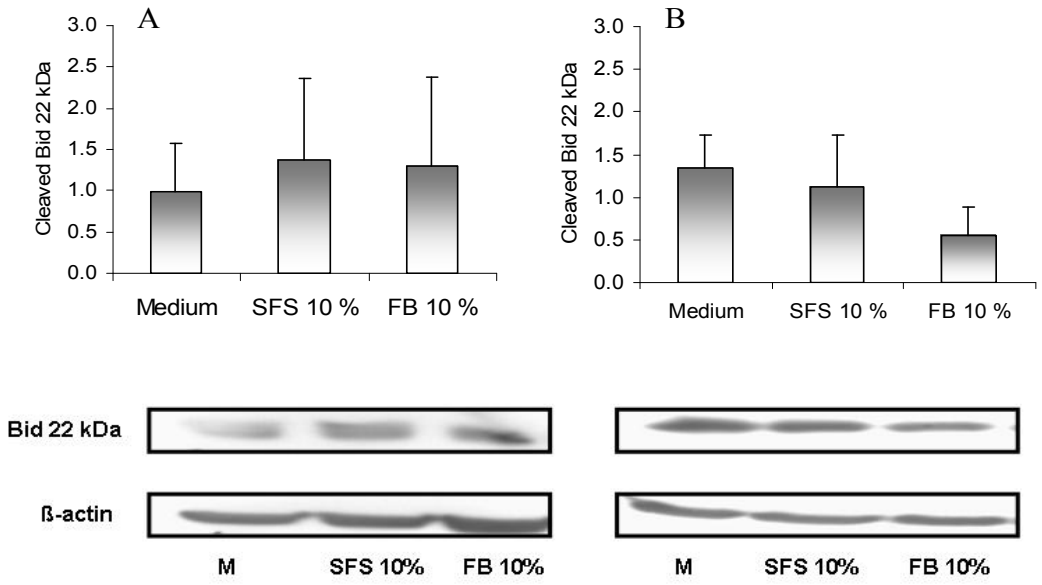


Figure 28 Effect of incubation with Synergy1 fermentation supernatant (SFS) and fermentation blank (FB) on Bid cleavage in LT97 (A) and HT29 (B) cells analysed with Western blot.

Displayed are the means and SD of 3 independently reproduced experiments. One way ANOVA, Bonferroni post-tests. (SFS 10 % Synergy fermentation supernatant 10 %, FB 10 %: Fermentation Blank 10 %)

In general, there was no significant change in the level of full length Bid (22 kDa) after incubating both the cell lines with 10 % SFS and FB after 24 h. These results correspond to the effects observed in the real time analysis. However, in HT29 cells a trend of decreased full length Bid was visible. This non-significant decrease in the amount of full length Bid from 1.34 to 1.12, HT29 cells could hint towards a weak signal of Bid induced apoptosis in HT29 cells.

4.11 Analysis of gene expression using custom array

Further, it was of interest to also analyse a broader range of genes involved in additional mechanisms of carcinogenesis or chemoprevention. This was done using a customised cDNA array system spotted with more than 300 genes of which apoptosis signaling pathway, tumour suppressor genes and cell cycle arrest/regulation of cell cycle were of specific interest (figure 29).

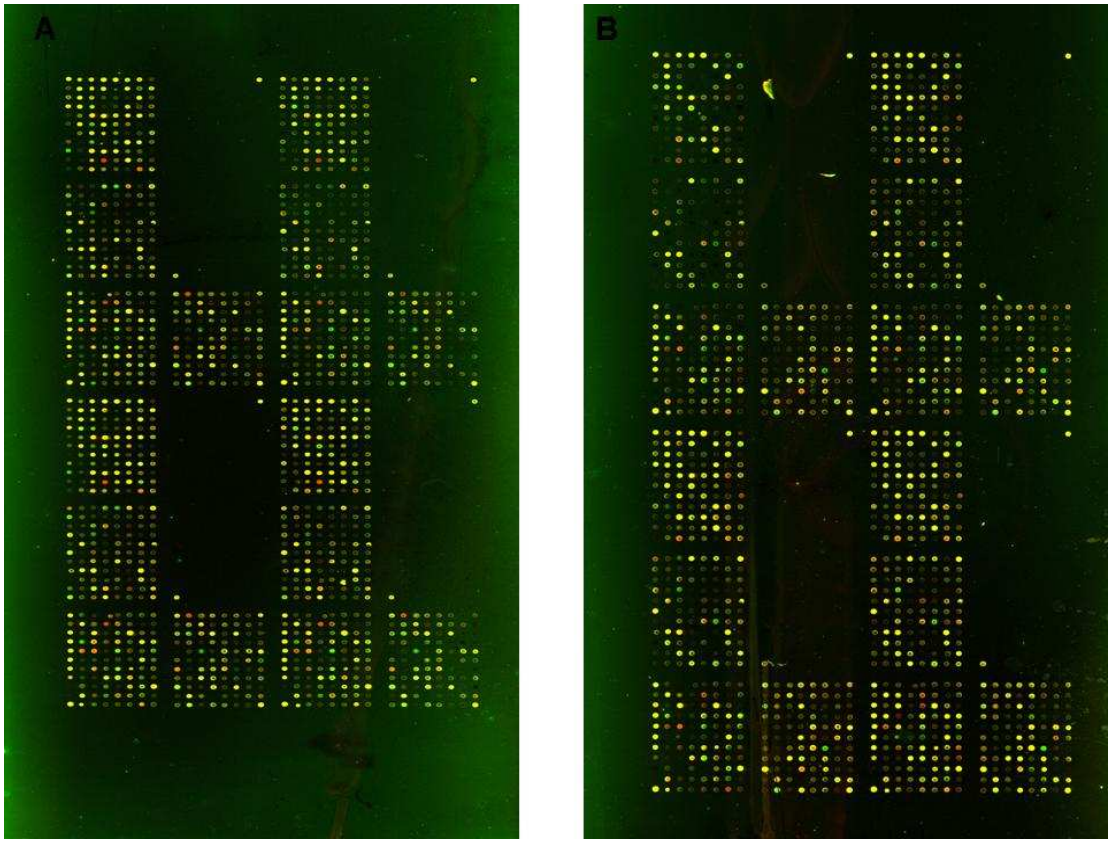


Figure 29 Representative of array image LT97 cells (A) and HT29 cells (B) after incubation with Synergy fermentation supernatant (SFS)

In LT97 cells; custom array results indicated that mRNA expression of 37 genes were up-regulated whereas 53 genes were down-regulated on the basis of the evaluation criteria (≥1.5 or ≤ 0.6) after incubation with SFS for 24 h. Among 37 genes found to be up-regulted; only 6 namely, Metallothionein 2A; MT2A (fold change 6.11), c-Fos proto-oncogene; FOS_2 (fold change 7.92), Heat shock 70 kDa protein 5; HSPA5 (fold change 3.80), DNA-damage-inducible transcript 3; DDIT3 (fold change 2.21) and two genes with miscellaneous functions; namely Aryl hydrocarbon receptor nuclear translocator; ARNT (fold change 1.77) and Baculoviral IAP Repeat-Containing Protein 4; BIRC4 (fold change 3.15) were found to be significantly up-regulated ($p \leq 0.05$, t test).

Among 53 genes found to be down-regulated after incubating LT97 cells for 24 h with SFS 10 %; 36 genes were significantly down-regulated on the basis of t-test. These include Acetyl-Coenzyme A acetyltransferase 2; ACAT2 (fold change 0.16), Epoxide hydrolase 2, cytoplasmic; EPHX2 (fold change 0.25), three genes of Glutathione S transferase family, GSTO1, MGST1, MGST2 (fold change 0.42, 0.27, 0.15 respectively), Catechol-Omethyltransferase; COMT (fold change 0.22), Sulfotransferase family, cytosolic, 1A; SULT1A1 (fold change 0.50), two UDP glucosyltransferase, UGT1A7, UGT2B17 (fold change 0.49, 0.57 respectively), two NRF1 mediated regulators KEAP1 and NRF2 (fold change 0.50 and 0.39 respectively), all seven genes related to oxidative and metabolic stress e.g. GPX1, GPX2, FMO5, HMOX1, HSPA1A, PRDX2, DIA4; two DNA damage repair genes namely XRCC2, XRCC5 (fold change 0.43, 0.47 respectively), two genes related to growth arrest CCND1, PCNA (fold change 0.45 and 0.32), Apoptosis signaling genes namely CASP3 and 6 (fold change 0.59 and 0.34), tumour necrosis factor receptor TNFSF6 and TNFSF10 (fold change 0.44 and 0.58), Bid (fold change 0.50), two cell cycle arrest genes; CCNB1, CCNB2 (fold change 0.40 and 0.53), tumour protein; PIG3 (fold change 0.35), two metal storage genes; FTL and SLC11A2 (fold change

0.60 and 0.31) and four genes with miscellaneous functions e.g. GSS, MCM7, NFKB1, TYMS (fold change 0.41, 0.23 and 0.31, 0.28) were down-regulated significantly (Table 4).

Table 4 Gene expression analysis of Synergy1 fermentation supernatant (SFS) treated LT97 cells using a custom made cDNA array

The table includes genes that have modulated expression levels of ≥ 1.5 and ≤ 0.6 fold compared to the medium control cells and are statistically significant. Significant differences to the medium controls were calculated with a two-tailed student t test ($p \leq 0.05$, n=3)

Gene name	Descriptions	Ratio	SD	P value	Sign	
Metallothioneins						
MT2A	Metallothionein 2A	6.11	2.74	0.003	**	↑
NF-E2 gene family NRF1 and 2 mediated regulations						
FOS_2	c-Fos proto-oncogene	7.92	4.37	0.03	*	↑
Stress and Signal transduction						
Oxidative or Metabolic Stress						
HSPA5	Heat shock 70kDa protein 5	3.8	1.32	0.02	*	↑
Growth Arrest and Senescence						
DDIT3	DNA-damage-inducible transcript 3	2.21	0.47	0.02	*	↑
Miscellaneous (several functions)						
ARNT	Aryl hydrocarbon receptor nuclear translocator	1.77	0.6	0.02	*	↑
BIRC4	Baculoviral IAP Repeast-Containing Protein 4	3.15	1.53	0.03	*	↑
Acetyltransferases						
ACAT2	Acetyl-Coenzyme A acetyltransferase 2	0.16	0.07	0.0007	***	↓
Epoxide Hydrolases						
EPHX2	Epoxide hydrolase 2, cytoplasmic	0.25	0.06	0.0002	***	↓
Glutathione S-Transferases						
GSTO1	Glutathione S-transferase omega 1	0.42	0.14	0.0019	**	↓
MGST1	Microsomal glutathione S-transferase1	0.27	0.11	0.01	*	↓
MGST2	Microsomal glutathione S-transferase 2	0.15	0.06	0.0015	**	↓
Methyltransferases						
COMT	Catechol-Omethyltransferase	0.22	0.08	0.001	**	↓
Sulfotransferases						
SULT1A1	Sulfotransferase family, cytosolic, 1A	0.5	0.17	0.01	*	↓

UDP Glycosyltransferases						
UGT1A7	UDP glycosyltransferase 1 family	0.49	0.18	0.03	*	↓
UGT2B17	UDP glycosyltransferase 2 family	0.57	0.18	0.02	*	↓
NF-E2 gene family NRF1 and 2 mediated regulations						
KEAP1	Kelch-like ECH-associated protein 1	0.5	0.17	0.01	**	↓
NRF2	Nuclear factor (erythroid-derived 2)-like 2	0.39	0.14	0.0047	**	↓
Stress and Signal transduction						
Oxidative or Metabolic Stress						
FMO5	Flavin containing monooxygenase 5	0.56	0.16	0.02	*	↓
GPX1	Glutathione peroxidase 1	0.46	0.15	0.02	*	↓
GPX2	Glutathione peroxidase 2	0.18	0.09	0.02	*	↓
HMOX1	Heme oxygenase (decycling) 1	0.48	0.24	0.05	*	↓
HSPA1A	Heat shock 70kDa protein 1A	3.8	1.32	0.02	*	↓
PRDX2	Peroxiredoxin 2	0.43	0.1	0.04	*	↓
DIA4	NAD(P)H Dehydrogenase	0.6	0.24	0.04	*	↓
DNA Damage and Repair						
XRCC2	X-ray repair complementing defective repair	0.43	0.08	0.0021	**	↓
XRCC5	X-ray repair complementing defective repair	0.47	0.14	0.01	*	↓
Growth Arrest and Senescence						
CCND1 (cyclin D1)	Cyclin D1	0.45	0.32	0.04	*	
PCNA	Proliferating cell nuclear antigen	0.32	0.17	0.01	**	↓
Apoptosis Signaling						
CASP3	Caspase 3	0.59	0.17	0.02	*	↓
TNFRSF6 (Fas)	Fas (TNF receptor superfamily, member 6)	0.44	0.18	0.02	*	↓
TNFSF10 (TRAIL)	Tumor necrosis factor (ligand) superfamily	0.58	0.13	0.03	*	↓
CASP6	CASPASE-6 Precursor	0.34	0.12	0.01	**	↓
BID: (BID)	BH3 interacting domain death agonist BID	0.5	0.13	0.03	*	↓
Cell Cycle Arrest-Regulation of cell cycle						
CCNB1	cyclin B1	0.4	0.23	0.03	*	↓
CCNB2	cyclin B2	0.53	0.23	0.02	*	↓
PIG3	tumor protein p53 inducible protein 3	0.35	0.17	0.04	*	↓
Metal storage and utilization						
FTL	Ferritin, light polypeptide	0.6	0.25	0.05	*	↓
SLC11A2 (DMT1)	Solute carrier family 11	0.31	0.18	0.05	*	↓
Miscellaneous (several functions)						
GSS	Glutathione synthetase	0.41	0.1	0.002	**	↓
MCM7	MCM7 minichromosome maintenance deficient 7	0.23	0.12	0.01	**	↓
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer	0.31	0.17	0.04	*	↓
TYMS	Thymidylate synthetase	0.28	0.24	0.03	*	↓

In contrast to SFS; incubation of FB resulted in up-regulation of 122 genes on the basis of our evaluation criteria but none of them was found to be statistically significant. However, among 12 genes genes down-regulted by FB in LT97 cells, four were found to be statistically significant namely Acetyl-Coenzyme A; ACTA2 and Epoxide hydrolase 2; EPHX2 were down-regulated with a fold change of 0.39 and 0.51 respectively, two genes with miscellaneous functions namely, minichromosome maintenance deficient; MCM7 and Thymidylate synthetase TYM8 were also down regulated significantly (fold change 0.28 and 0.35 respectively) (Table 5). Detailed description of all the genes modulated by SFS and FB in LT97 cells are listed in Appendix E.

Table 5 Gene expression analysis of fermentation blank (FB) treated LT97 cells using a custom made cDNA array

The table includes genes that have modulated expression levels of ≥ 1.5 and ≤ 0.6 fold compared to the medium control cells and are statistically significant. Significant differences to the medium controls were calculated with a two-tailed student t test ($p \leq 0.05$, n=3)

Gene name	Descriptions	Ratio	SD	P value	Sign	
Acetyltransferases						
ACAT2	Acetyl-Coenzyme A	0.39	0.2	0.01	*	↓
Epoxide Hydrolases						
EPHX2	Epoxide hydrolase 2	0.51	0.28	0.04	*	↓
Miscellaneous (several functions)						
MCM7	minichromosome maintenance deficient	0.28	0.14	0.02	*	↓
TYMS	Thymidylate synthetase	0.35	0.08	0.0003	***	↓

In HT29 cells custom array results also indicated significant up regulation of 7 genes out of total 97 genes up-regulated on the basis of evaluation criteria (≥ 1.5 or ≤ 0.6) after 24 h incubation with SFS. The significantly up-regulated genes were sulfotransferase; SULT1C1 (fold change 3.12), Metallothionein 2A; MT2 A (fold

change 2.56), Transcription Factor JUND (fold change 2.64), cyclin A; CCNA1 (fold change 1.86), Cyclin-dependent kinase inhibitor2B; CDKN2B (fold change 3.03), Ferritin, light polypeptide; FTL (fold change 2.21), and one gene with miscellaneous function namely Aryl hydrocarbon receptor nuclear translocator; ARNT (fold change 2.59) was also significantly altered.

Among the 28 genes down-regulated by SFS in HT29 cells 6 genes were down-regulated significantly e.g. Methyl transferases; NNMT (fold change 0.58), DNA damage and repair gene; ERCC6 (fold change 0.43), Proliferating cell nuclear antigen; PCNA (fold change 0.26), Haeamochromatosis; HFE (fold change 0.54) and two genes with miscellaneous functions namely MCM7 minichromosome maintenance deficient; MCM7 and Nuclear factor of kappa light polypeptide gene enhancer; NFKB1 (fold change 0.39 and 0.35 respectively) (Table 6).

Table 6 Gene expression analysis of Synergy1 fermentation supernatant (SFS) treated HT29 cells using a custom made cDNA array

The table includes genes that have modulated expression levels of ≥ 1.5 and ≤ 0.6 fold compared to the medium control cells and are statistically significant. Significant differences to the medium controls were calculated with a two-tailed student t test ($p \leq 0.05$, n=3)

Gene name	Descriptions	Ratio	SD	P value	Sign	
Sulfotransferases						
SULT1C1	Sulfotransferase family, cytosolic, 1C, member 1	3.12	2.77	0.04	*	↑
Metallothioneins						
MT2A	Metallothionein 2A	2.56	1.21	0.04	*	↑
CCAAT-enhancer-binding protein (C/EBP) mediated regulation						
JUND	Transcription Factor JUND	2.64	0.37	0.003	**	↑
Cell Cycle Arrest-Regulation of cell cycle						
CCNA1	cyclin A	1.86	0.36	0.04	*	↑
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	3.03	0.76	0.03	*	↑
Metal storage and utilization						
FTL	Ferritin, light polypeptide	2.21	0.24	0.02	*	↑
Miscellaneous (several functions)						
ARNT	aryl hydrocarbon receptor nuclear translocator	2.59	1.52	0.05	*	↑

Methyltransferases						
NNMT	Nicotinamide Nmethyltransferase	0.58	0.18	0.02	*	↓
DNA Damage and Repair						
ERCC6	Excision repair cross-complementing rodent repair deficiency	0.43	0.06	0.04	*	↓
Growth Arrest and Senescence						
PCNA	Proliferating cell nuclear antigen	0.26	0.14	0.0036	**	↓
Metal storage and utilization						
HFE	Hemochromatosis	0.54	0.29	0.04	*	↓
Miscellaneous (several functions)						
MCM7	MCM7 minichromosome maintenance deficient	0.39	0.24	0.02	*	↓
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer	0.35	0.04	0.05	*	↓

Incubation with FB resulted in an up-regulation of 44 genes but similar to LT 97 cells none of the up-regulated gene was significantly modulated. However, one of the five down-regulated genes namely Uracyl DNA glycolase was significantly down-regulated with a fold change 0.57 in HT29 cells after incubation with FB (Table 7).

Table 7 Gene expression analysis of fermentation blank (FB) treated HT29 cells using a custom made cDNA array

The table includes genes that have modulated expression levels of ≥ 1.5 and ≤ 0.6 fold compared to the medium control cells and are statistically significant. Significant differences to the medium controls were calculated with a two-tailed student t test ($p \leq 0.05$, $n=3$)

Gene name	Descriptions	Ratio	SD	P value	Sign	
DNA Damage and Repair						
UNG	Uracil-DNA glycosylase	0.57	0.07	0.04	*	↓

5 Discussion

Tumourigenesis has long been thought to be a multistep process [Foulds, 1958]. However, only recently it has become possible to identify the molecular events that underlie the initiation and progression of human tumours. Large bowel cancer, especially in colon and rectum, is a leading cause of cancer mortality in the industrialised world [Dove-Edwin and Thomas, 2001]. The development of colorectal cancer is complex process, involving both environmental and genetic factors. However, casual associations obtained in epidemiological studies between large bowel cancer development and diets are controversial and still a subject to debate.

Dietary fibres have been put forward as colon cancer protective food components [Burkitt, 1969; Hill, 1995]. The theory is that dietary fibre may protect against colon cancer through secondary events resulting from the fermentation of carbohydrates by the gut microflora. This leads to faecal bulking, increased speed of colonic transit, increase in nitrogen metabolism, increased bacterial load in the colon, acidification and, finally the production of SCFA [Harris and Ferguson, 1993]. Support for this concept comes from various animal and cell culture experiments reviewed earlier [Johnson, 1995; Velazquez et al., 1996]. One class of dietary fibres that could be excellently relevant in colon cancer prevention is the group of inulin-type fructans. Earlier studies showed a reduction of crypt numbers and multiplicity, when adding 10 % inulin to the diet of rats [Rowland et al., 1998]. Accumulating evidence for a preventive effect of inulin against colon cancer in experimental animals has been reviewed in detail [Pool-Zobel, 2005; Pool-Zobel and Sauer, 2007]. These animal studies have shown that a functional intake of inulin type fructans is the inhibition of carcinogen mediated tumour formation. However, identifying potential anticancer properties of chemopreventive substances using animal models is time consuming and

expensive. Therefore, the present study was performed using *in vitro* cell culture approaches to elucidate in depth which functional consequences can ensue from inulin type fructans in human colon with regard to chemoprotection on the cellular level. For this, a fermentation supernatant simulating the conditions of the intestinal lumen was produced *in vitro* and used to treat human colon tumour cell lines to determine effects on diverse parameters of chemoprotection. These include parameters related to primary cancer prevention (which prevent cancer initiation by blocking carcinogens from damaging the cells) and secondary chemoprotection (neoplastic cells are suppressed from further progression).

5.1 Fermentation of inulin type fructans

Inulin enriched with oligofructose (Synergy1) is a source of prebiotic dietary fibre which yields high amounts of SCFA due to fermentation by gut bacteria [Sauer et al., 2007b]. These SCFAs are able to reduce the conversion of primary to secondary bile acids in the colon [Bingham, 1996]. Our results indicate a 2.6 fold increase in SCFA and 3.4 fold reduction in the amount of DCA in SFS as compared to FB after *in vitro* fermentation of Synergy1. The total amounts of SCFA after fermentation of Synergy1 (126 mM) observed in this study correspond to normal situation as the total amount of SCFA observed *in vivo* in the colon of human is estimated to range from 70-140 mM [Reddy, 1999]. Noteworthy, the absolute concentration of butyrate observed in the present study increased from 6.6 mM in FB to 26.4 mM in SFS accounting for almost 4 fold increase. Additionally, the relative molar concentrations, with molar ratios for acetate, propionate and butyrate of FB (62:22:16) and SFS (65:14:21), also indicate a particular modified profile of propionate and butyrate. Thus, high production of SCFA *in vitro* is possibly also an indication of protective effects, because butyrate and propionate, have antiproliferative properties in colon tumour cells [Beyer-Sehlmeyer

et al., 2003]. Moreover, the analytical measurements of bile acids revealed a concentration of 12.3 mM in FB and 3.6 mM in SFS which are similar to the previous results with fiber rich diet [Alberts et al., 2003; Owen, 1997]. These results are noteworthy because bile acids have been shown to positively correlate with colon cancer incidence (Bernstein, 2005). Further, increased level of SCFA produced during fermentation reduces luminal pH and is responsible for a decreased conversion of primary to secondary bile acids [Bingham, 1996]. Therefore, these results indicate that fermentation of Synergy1 after ingestion may be inversely associated in terms of incidence of colon cancer.

5.2 Growth inhibitory effects

Inulin and oligofructose (Synergy1) has been the subject of various *in vitro* and *in vivo* studies [Roberfroid, 1993; Pool-Zobel, 2005]. It has been shown previously that gut fermentation products of inulin-type fructans beneficially modulated markers of tumour progression in human colon tumour cells [Klinder et al., 2004b]. Furthermore, *in vivo* studies also showed the incidence of carcinogen mediated colonic tumours reduced after life-long feeding of 10 % inulin to rats [Verghese et al., 2002]. Thus, these studies have shown that a functional result of consumption of Synergy1 is the inhibition of tumour formation.

On the basis of our results, we found that fermentation of Synergy1 resulted in a distinct time and concentration dependant inhibition of cell growth in both cell lines. The reduction of cell growth *in vitro*, by fermentation samples in this study are in line with a similar previous *in vitro* [Klinder et al., 2004b] and *in vivo* studies [Femia et al., 2002]. Among SCFA, butyrate has been shown to be a potent inhibitor of cell growth. However, the impact on cell proliferation is additive with propionate in complex fermentation supernatants [Scheppach et al., 1995]. In cultured cell lines,

butyrate is a well recognised antiproliferative agent arresting cell growth in G_1 and inducing differentiation [Wong et al., 2006]. We also observed an almost four fold increase in butyrate concentration after *in vitro* fermentation of Synergy1 which may contribute to the significant growth inhibition in both cell lines. Moreover, the distinct time dependency of inhibition of proliferation with SFS supports the production of effective metabolites produced during incubation of fermentation supernatant with colon cells [Klenow et al., 2008], which in turn could induce intracellular signal cascades. Interestingly, the fermentation sample from Synergy1 and its corresponding synthetic mixture consisting of acetate, propionate, buyrate and DCA were of equal potency in mediating growth inhibition, reflecting the potential of SCFA in secondary chemoprevention. Furthermore, our data showed that the growth inhibitory effect of SFS is stronger in adenoma than in carcinoma cells. This difference in growth inhibition may potentially be explained by differences in the amount of butyrate uptake by both cell lines, which may be caused by the availability of monocarboxylate transporter 1 (MCT1) which is necessary for butyrate uptake and is down-regulated from normal cells to malignant cells [Daly et al., 2005;Cuff et al., 2002]. Therefore, adenoma cells which represent an early stage of cancer might have consumed more butyrate and in turn their growth could be inhibited in a stronger manner in comparison to carcinoma cells. Altogether, this may have important implications on chemoprevention, as it indicates that fermentation products of Synergy1 could inhibit growth of adenoma cells, thus acting at an early stage of carcinogenesis and reducing the probability of the formation of malignant tumours. Additionally, the increase in metabolic activity of remaining cells after 72 h with SFS pointed towards nutrient effects and indicated that the remaining cells were metabolically more active after treatment with SFS. Moreover, increase in growth observed in HT29 cells after incubation with lower concentrations of SFS and FB

could be due to the presence of secondary bile acids which in small concentrations have been shown to increase the proliferation of HT29 cells [Milovic et al., 2002]. This increase in cellular growth was not visible with higher concentration of SFS or FB which may be due to the detoxification of secondary bile acids with SCFAs. Furthermore, since incubation of cells with FB also reduced cell growth, additional factors seem to be present in the feces supernatant bearing an effect on cells. Factors like sulphates, ammonia and products of bacterial metabolism, non-digested food residues and excretable metabolites could account for these effects [Cummings and Englyst, 1987].

5.3 Modulation of genotoxicity

Although the colon epithelium is partly protected by its mucin layer, which can detoxify reactive substances, yet the cells are relatively susceptible to exogenous toxins. Compared to liver, they express only relatively small amounts of phase II enzymes, which inactivate many genotoxic carcinogens [Berlau et al., 2004]. Therefore, the possible causes of colorectal cancer could also be due to the lack of antioxidative potential of colonic mucosa. In the present study, physiologically relevant concentrations of H_2O_2 and HNE were used as model genotoxic substances. Both of these substances generate oxidative stress in the form of reactive oxygen species (ROS), which lead to experimentally induced DNA damage [Glei et al., 2006]. Pre-treating HT29 cells with SFS for 24 h, resulted in a significant decrease in DNA damage induced by subsequent H_2O_2 challenge whereas no such effect was visible in LT97 cells. This SFS treatment did not affect viability of both cell lines which demonstrates the absence of cytotoxic effects. The current findings thus disclose that SFS protects DNA of HT29 cells from H_2O_2 induced DNA damage. This could be a reflection of enhanced cellular metabolism, including stimulation of DNA repair and

antioxidant defence sytems e.g. up-regulation of catalase, which in turn is responsible for decomposing H_2O_2 [Abrahamse et al., 1999]. In this context the antigenotoxic properties of SCFA especially butyrate and acetate are of special importance as both compounds are utilized by colonocytes as energy source [Clausen and Mortensen, 1995]. However, the decrease in genotoxicity could not be confirmed for the prevention of HNE induced genotoxicity which may require a higher amount of SCFA especially butyrate or longer incubation times to reach this functional consequence. Additionally, in LT97 cells, SFS was unable to modulate the genotoxic effects of both model genotoxins which might be due to up-regulation of genes responsible for detoxification in HT29 cells.

5.4 Effect on mRNA expression of GSTA4 and CAT and subsequent Enzyme activities

In the present study, we observed a significant increase in GSTA4 mRNA expression in HT29 cells after preincubation with SFS. The products of this gene is known to protect against oxidative stress, endogenous aldehydes, quinones, epoxides and hydroperoxides formed as secondary metabolites during oxidative stress and can therefore protect from food contaminants, such as polycyclic aromatic hydrocarbons [Hayes and Pulford, 1995]. Since GSTA4 is the GST isoenzyme with the highest affinity for HNE, the lack of protection against HNE induced DNA damage was rather surprising and could be explained due to post transcriptional modification of this gene. Furthermore, a significant induction of GSTA4 mRNA after pretreatment of cells with faecal blank indicates the protective effects for e.g. products of bacterial metabolism of nondigested food residues and excretable metabolites which may have their origin from faecal samples rather than from fermented Synergy1 which need to be investigated in detail in further studies.

Our results raise interesting questions regarding the mechanisms of GST mRNA induction in human colon cancer cells. The putative binding sites for transcription factors in the regulatory region of GSTA4 gene include AP1, STAT, and NFkB [Desmots et al., 1998]. In addition, butyrate activates ERK of the MAP kinase pathway which represents an important component of induction of the AP1 related transcription factors Jun and Fos [Ebert et al., 2001]. However, it must also be considered that our model (HT29 cells) represents transformed cells and that the dietary fibres mediated up-regulation of GSTA4 mRNA may potentially enhance survival of transformed cells. Accordingly, additional work is needed on how the relative mRNA expression levels in human primary cells may be influenced by dietary fibres which in turn could confer protection of these cells against transformation. In contrast to HT29 cells, no change in GSTA4 mRNA was observed in LT97 cells which could however be due to less expression of GSTs in LT97 cells in comparison to HT29 cells [Schaeferhenrich et al., 2003].

At the enzymatic level, GSTs constitute the major family of phase II detoxification enzymes involved in the cellular protection against electrophilic intermediates by catalysing conjugation with GSH [Habig et al., 1974]. Accordingly, elevation of GST and GSH expression by chemopreventive agents can afford cellular protection against carcinogens. In this regard, several studies have provided evidence that GST isoenzymes may be induced by nutritional factors *in vivo* and *in vitro* [Knoll et al., 2005; Pool-Zobel, 2005]. It has been shown previously in human colon cells that exposure to 4 mM butyrate induces total GST and catalytic activity [Ebert et al., 2001]. However, we found no change in GST activity in both the cell lines after 24 h of treatment with SFS corresponding to a previous *in vitro* study [Beyer-Sehlmeyer et al., 2003]. This could be either explained by too low butyrate concentrations (1.3 mM)

in the SFS; since butyrate induces GST activity at 4 mM [Glei et al., 2006] or by the presence of other inhibitory substances in the faeces.

In addition to GSTs, catalase is also a phase II enzyme which represents one of the key defense systems against oxidative stress because it detoxifies H_2O_2 to oxygen and water [Chelikani et al., 2004]. A previous study showed a significant increase in the mRNA expression of the catalase gene (2.9 fold) and a 65 % increase in its enzymatic activity after incubation of primary colon cells of humans with 10 mM butyrate for 2h [Sauer et al., 2007a]. In the present study, only a non-significant increase in the mRNA expression of catalase was observed (fold change 1.85) but a significant 2 fold increase in catalase activity after incubation of carcinoma cells with SFS for 24h. This significant up-regulation of catalase activity suggests that catalase activity may not be regulated on the transcriptional level but by some yet unknown post translational mechanisms. Moreover, since catalase decomposes H_2O_2; induction of catalase can protect the cells against endogenous H_2O_2 induced DNA damage, risk of mutations and possibly initiation of cancer [Mates and Sanchez-Jimenez, 2000].

5.5 Induction of Apoptosis

In addition to cell growth inhibition, induction of apoptosis in cancer cells is another important mechanism by which tumours can be prevented to grow further. Apoptosis, is a physiological process of selected cell deletion and is thus important for secondary cancer prevention, if activated in cancer cells [Kerr et al., 1972]. If apoptosis is suppressed (e.g. in cells with p53 mutations) this can result in the development of various tumours [Hollstein et al., 1991]. Cells committed to terminal differentiation undergo a genetically programmed death process that exhibits features of apoptosis such as cell shrinkage and nuclear DNA fragmentation [Hall et al., 1994]. Previous studies have shown that the Bcl-2 family of proteins may play a critical role

in regulating cell fate in the intestinal epithelium [Kapiteijn et al., 2001]. Furthermore, both Bcl-2 and Bcl-XL, two antiapoptotic members of Bcl-2 family, have been found to be overexpressed in colon cancer [McEntee et al., 1999; Maurer et al., 1998]. Therefore, to study the impact of inulin type fructans on apoptosis, as a relevant mechanism for anti cancer properties of dietary fibres, colon adenoma and carcinoma cell lines were incubated with SFS and the effect on expression of proapoptic genes at mRNA and enzymatic level were investigated.

5.6 Induction of PARP cleavage

To analyse effects of SFS and FB on apoptosis, cleavage of Poly (ADP-ribose) polymerase (PARP) was measured. Inhibition of PARP by cleavage facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [Germain et al., 1999]. In general, we observed a significant increase in cleaved PARP after incubating the adenoma cells with SFS. The association of PARP function with cell death is inferred from studies demonstrating that PARP is rapidly and specifically cleaved during apoptosis [Tewari et al., 1995; Lazebnik et al., 1994]. PARP has been proposed to act as a critical death substrate, because it is rapidly cleaved by caspase-3, a mammalian ICE-related protease involved in apoptosis [Nicholson et al., 1995]. Therefore, the observed significant increase in PARP cleavage indicates that dietary fibers may have beneficial chemopreventive effects by inducing apoptosis in colon cancer cells. The mechanism by which inulin type fructans induce PARP cleavage is still unclear. It has been shown previously that butyrate induced apoptosis is mediated via activation of the caspase-cascade and a key event was the proteolytic activation of caspase-3, triggering degradation of PARP [Ruemmele et al., 1999]. Furthermore, we were able to show that SCFA alone also significantly increased apoptosis in both cell lines. However, when SFM (containing SCFA and DCA) was

used, induction of apoptosis was less pronounced. DCA has been shown previously to cause degradation of p53 [Bernstein et al., 2005] which in turn increases genomic instability because of reduced apoptotic removal of cells with DNA damage. Thus, DCA might have inhibited the apoptosis inducing effect of SCFA when present together with SCFA [McMillan et al., 2000]. *In vivo* both SCFA and bile acids are present together in the colon so this apoptosis inhibiting effect of bile acids could be significant in colonic tissue which turns over very rapidly. Moreover, in comparison to SFM, the complex SFS induced apoptosis in a stronger way which is a hint towards the role of some other not yet identified substances. The so called "added value" of the complete fermentation samples could be due to an increase in antioxidants and anti-mutagenic compounds like hydroxycinnamic acids which occur in cell walls of some species of food plants and may be released after microbial fermentation [Ferguson and Harris, 2003]. As a result, such compounds could, at least theoretically, inhibit the development of colon cancer. Additionally, the increased sensitivity of LT97 cells to be triggered to go into apoptosis may have important implications for possible chemopreventive activities in earlier stages of the cancer process, because it is of high benefit to induce apoptosis in adenoma cells and thereby reduce the formation of more degenerated carcinoma cells. Altogether, the investigation revealed a further important mechanism of secondary chemoprevention by complex fermentation samples by induction of apoptosis. This effect was primarily mediated by SCFA and suppressed by DCA.

5.7 Modulation of Caspase activity

In addition to PARP cleavage, most apoptosis signaling pathways ultimately result in the activation of caspases, a family of cysteine proteases that act as common death effector molecules in various forms of death [Degen et al., 2000]. Activation of

caspases can principally be triggered by at least two distinct, but interconnected pathways that activate different initiator caspases, that is caspase-8 and -9 which converge at the level of executor caspase -3, -6, and -7 [Li et al., 1998]. The simultaneous activation of both pathways is thought to result in a strong amplification of original apoptosis signal. Therefore, activity of different caspases was measured in order to get more information on the mechanisms of how the complex fermentation sample induced apoptosis. Here, we observed that treatment of both carcinoma and adenoma cell lines with SFS and SFM induced the activation of caspase-3, which is consistent with the previous studies where apoptosis was induced by short chain fatty acids especially butyrate [vivi-Green et al., 2000; Mandal et al., 2001]. This observation is also in accordance with the results obtained from PARP cleavage studies whichis is known to be a substrate of caspase-3 [Yang et al., 2001]. Caspase-3, a member of executior caspases [Kumar S, 2007] can be activated by several routes which include ligation of cell surface receptors such as Fas which are associated with caspase-8; or by factors released from mitochondria, that is cytochrome c, in response to cellular stress, which in turn activate caspase-9 [Enari et al., 1996]. It has been recently shown recently that sodium butyrate could induce the conversion of the proenzyme form of caspase-3 to the catalytically active effector protease through histone deacetylase inhibition which sensitises tumour cells to induction of apoptosis [Medina et al., 1997]. Beside Caspase-3; Caspase-8 and -9 were also investigated after incubation of both cell lines with SFS and FB. However, incubation of both cell lines with SFS or FB resulted in either no or non-significant increase in cleavage of caspase -8 and-9. Recently, studies have identified caspase-10 as an important initiator caspase, in addition to caspase-8 and-9, in death receptor signaling pathways [Kischkel et al., 2001]. Therefore, factors other than caspase-8 and -9 e.g. caspase-10

or caspase-1 [Harvey et al., 1998] may play an important role as the major executer together with caspase-3 in SFS-induced colon cancer cells apoptosis.

5.8 Modulation of Apoptosis relevant genes

In addition to involvement of caspases recent studies have begun to dissect the molecular mechanisms by which apoptosis is induced in tumour cells. In this regard, Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is an attractive candidate for cancer prevention due to its ability to induce apoptosis selectively in cancer cells [Walczak et al., 1999; Lawrence et al., 2001]. Death receptor 4 (DR4) and Death receptor 5 (DR5) are members of the TNF receptor family and are receptors for TRAIL. DR5 and DR4 mediate TRAIL-induced apoptosis through the formation of a death-inducing signaling complex (DISC) containing the death receptor, adaptor proteins, such as Fas-associated death domain (FADD), and certain initiator caspases, such as procaspase-8 [Ashkenazi and Dixit, 1998] or procaspase-10 [Kischkel et al., 2001]. Furthermore, proteins of Bcl-2 family also play a pivotal role in the regulation of apoptosis. The family Bcl-2 can be subdivided into pro-and anti-apoptotic proteins. The anti apoptotic members include Bcl-2, Bcl-XL whereas the proapoptotic members include Bak, Bax, Bid [Cory and Adams, 2002].

The results of this study demonstrated a significant increase in mRNA expression of DR5 and Bax in HT29 colon carcinoma cells after incubation with SFS. Death receptors DR4 and DR5 have been demonstrated previously to play a crucial role in synergistic cytotoxicity with TRAIL and butyrate to induce apoptosis [Hernandez et al., 2001]. It has been postulated that the interaction of TRAIL with death receptors DR4/DR5 activates the caspase cascade, which in turn results in apoptosis [Walczak and Krammer, 2000]. However, TRAIL can induce apoptosis through DR4 and DR5 independently, but at physiological conditions it binds with a higher affinity to DR5

than to DR4 [Truneh et al., 2000]. In contrast to HT29 cells this increase in DR4 and DR5 receptors was not observed in LT97 cells. Although this has not been studied very well, there are indications that in some cell lines, these death receptors are more efficient in death signalling than the other [van Geelen et al., 2004]. Moreover, a different redistribution of TRAIL receptors to the cell membrane of LT97 cells after treatment with SFS might be responsible for effects observed in this study.

Furthermore, in addition to TRAIL receptors; Bax a proapoptotic member of the Bcl2 has also been shown to be one of the major players in mitochondria-mediated cell apoptosis either by down-modulation of Bax-antagonists such as Bcl-xL [Vander Heiden and Thompson, 1999] or by cytochrome c release from mitochondria. It has been proposed that after stimulation with butyrate the mitochondrial Bcl-2 is shifted towards a proapoptotic constellation, with markedly increased mitochondrial bak and bax levels and decreased bcl-xL levels. This shift allows the translocation of cytochrome-c from the mitochondria to the cytosol, allowing the formation of an apoptosome and upregulating caspase cascade leading to apoptotic cell death [Ruemmele et al., 2003]. Thus, results of the present study suggest that fermentation supernatant induced apoptosis is mediated by both TRAIL and Bcl-2 route in HT29 cells. Furthermore, these results also suggest that a combination of HDACIs and TRAIL thus might be useful as a promising strategy for cancer therapeutics and prevention. However, the non-observance in the up-regulation of TRAIL receptors and Bcl-2 members does not rule out the possibility to enhance TRAIL-induced apoptosis by regulating the expression of other proteins pertinent to the extrinsic or intrinsic pathway in LT97 cells. Further, the increase in TRAIL sensitivity which occurs during transition from an adenoma to a carcinoma stage could also be responsible for TRAIL induced apoptosis observed in HT29 cells. [Hague et al., 2005]

In addition our investigation also suggests no changes of Bid either at mRNA level or at protein level in both cell lines. Bid is a unique BH3 only proapoptotic protein and unlike others activation of Bid depends on the proteolytic processing of intact Bid into truncated Bid which in turn leads to a release of apoptogenic molecules such as cytochrome c [Luo et al., 1998]. In contrast to the present study a significant increase in Bid cleavage was observed in LT97 cells after incubation with 10mM butyrate for 24 h (Scharlau et al. unpublished). This could however be explained by either too low butyrate concentration in our fermentation supernatant or inhibition of Bid cleavage by other members of Bcl-2 family [Yi et al., 2003].

5.9 Analysis of other genes relevant to growth inhibition and apoptosis by custom array

We used a custom array with more than 300 genes with a known identity to analyse mRNA expression of genes after SFS treatment. In general, in both cell lines the expression of the majority of genes remained unchanged following treatment with SFS. However, each cell line had a distinctive expression profile in response to SFS treatment.

LT97 cells

In LT97 cells SFS treatment lead to an up-regulation of 37 genes. Out of these genes only 6 were found to be significantly modulated. The significantly upregulated genes included metallothioneins MT2A and a FOS gene family member FOS_2, Heat shock protein HSPA5, DNA-damage-inducible transcript 3; DDIT3 and two genes with miscellaneous functions; namely ARNT and BIRC4.

Metallothioneins belong to a family of cysteine-rich low molecular weight metal binding proteins and participate in detoxification of toxic metals and help in protection of cells against reactive oxygen species [Nordberg, 1998; Yi et al., 2003]. Furthermore, the, Fos gene family proteins have been implicated as regulators of cell proliferation, differentiation and transformation [Sugio et al., 1988] and considered to be good prognostic marker for patients with colorectal carcinoma. Additionally, DDIT3 has been shown to act in response to the stress of chemical exposure and other events blocking the proliferation at G1 and G2 check points [Fornace, Jr. et al., 1989]. It also binds to various transcription factors, resulting in induction of apoptosis.

Incubation of SFS also resulted in a down-regulation of 53 genes of which 36 genes were significantly modulated. These include ACAT2; EPHX2, three genes of Glutathione S transferase family, GSTO1, MGST1, MGST2; COMT; SULT1A1 two UDP glucosyltransferase, UGT1A7, UGT2B17; KEAP1 and NRF2; all seven genes related to oxidative and metabolic stress e.g. GPX1, GPX2, FMO5, HMOX1, HSPA1A, PRDX2, DIA4; two DNA damage repair genes namely XRCC2, XRCC5 respectively, two genes related to growth arrest CCND1, PCNA; apoptosis signaling genes namely CASP3 and 6; TNFSF6 and TNFSF10; Bid two cell cycle arrest genes CCNB1, CCNB2; tumour protein; PIG3; two metal storage genes; FTL and SLC11A2 and four genes with miscellaneous functions namely: GSS, MCM7, NFKB1, TYMS

Among growth inhibitory genes Cyclins are important as they constitute a group of proteins which are potentially synthesized and degraded during the cell cycle. This enables them to activate the cyclin dependant kinases at appropriate times whose activity is needed to drive the cells through the cell cycle [Desdouets et al., 1995]. Furthermore, the role of PCNA in cell cycle control is related to its interaction with cyclin [Paunesku et al., 2001]. Therefore, the down-regulation of cyclin A and PCNA mRNAs in cells treated with SFS indicates that SFS negatively affected cyclin A and

PCNA gene transcription. Furthermore, decreased CCNB1 and CCNB2 after incubation with SFS could impair CDK1 activation altering mitotic progression [Berger et al., 2006].

In contrast to SFS, treatment of FB did not result in any significant up/down-regulation of any of the growth arrest or apoptosis inducing genes.

HT29 cells

SFS treatment leads to a significant up-regulation of 97 genes in HT29 cells. However, only 7 were found to be statistically significant which include SULT1C1, MT2 A, JUND, CCNA1, CDKN2B, FTL, and ARNT. It has been shown previously that activation of cyclin A which drives the cells into cell cycle by activated cAMP is also accompanied by inhibition of cyclin D1 expression [Desdouets et al., 1995]. The growth factor induced cyclin D expression is repressed upon treatment with cAMP analogues, thus leading to an arrest during the G0/G1 transition [Sewing et al., 1993]. Therefore, induction of both CCNA1 after incubation with SFS seems to be positive in terms of chemoprevention.

Moreover, transcription factor JunD is highly expressed in intestinal epithelial cells, but its exact role in maintaining the integrity of the intestinal epithelial barrier remains unknown. Although *c-jun* and *junB* behave as immediate early response genes and enhance the G1 to S phase transition of the cell cycle upon mitogenic stimulation [Ryder et al., 1988] yet the function of JunD is much less clear and several studies have suggested that it may actually function to inhibit cell proliferation. Thus these findings are partially consistent with the growth inhibitory effect of SFS in HT29 cells. However, the contribution of signalling pathway to cell growth may depend on both cell type and cell cycle status which should be also taken into

consideration in further investigation. Furthermore, incubation of HT29 cells with SFS also resulted in a non-significant up-regulation of caspase-8,-9 which is consistent with our real time PCR results.

Among 28 genes down-regulated by SFS in HT29 cells, only 6 were found to be significantly modulated, which included NNMT, ERCC6, PCNA, HFE and two genes with miscellaneous functions namely MCM7 and NFKB1. Of all these 6 genes only PCNA has been thought to be responsible to inhibit growth of tumour and inhibit apoptosis (Paunesco 2001) and therefore could serve as a possible candidate for chemoprevention if down-regulated at mRNA level. Furthermore, NNMT has also been identified as a novel serum marker for human colorectal cancers [Roessler et al., 2005] and the up-regulation of NNMT has been found to be positively correlated with colorectal cancer and is required during initial phase of malignant conversion. [Kim et al., 2009].Therefore, these results hint that SFS might reduce the probability of colorectal tumour progression. Similar to LT97 cells, there was no significant up-regulation/down-regulation of genes related to growth inhibition or apoptosis induction after treatment of FB.

Collectively our results demonstrate marked heterogeneity between adenoma and carcinoma cell lines in the responsiveness of growth inhibition and apoptosis gene induction which could be due to the difference in availability of fermentation products in both cells lines or to the significant differences between adenoma and carcinoma cells in general. However, these results should be validated with real time PCR and at the enzymatic level and supported by *in vivo* studies, especially in human intervention trials.

6 Abstract

Introduction Epidemiological studies suggest that a diet rich in fat and red meat may increase the risk of colon cancer, whereas a high intake of fibre and complex carbohydrates may protect against it. One of the potentially protective groups of dietary constituents are prebiotics which are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of certain groups of beneficial bacteria while maintaining potential pathogens at low levels. Among various prebiotics inulin and oligofructose has been the subject of extensive research. Inulin type fructans are the natural constituents of a wide range of common vegetables and fruits. Potential fermentation products of inulin in the gut include SCFA, especially an increased relative proportion of butyrate. These fermentation products are shown to be protective in different stages of cancer onset since they regulate colonic epithelial turnover and induce apoptosis in colon adenoma and cancer cell lines.

Aims The aim of this study was to characterise the fermentation supernatant of Synergy1 (SFS) for parameters of chemoprotection, mainly inhibition of tumour cell growth, induction of apoptosis and modulation of genetic damage. Moreover, we compared functional consequences of this supernatant using two different colon cancer cell lines namely LT97 (preneoplastic adenoma cells) and HT29 (highly transformed adenocarcinoma cells) to obtain new information on the molecular mechanisms of tumour suppression. Furthermore, a synthetic fermentation mixture (SFM) mimicking the SFS in the amount of short chain fatty acids (SCFA) and the secondary bile acid desoxycholic acid (DCA) was also prepared to investigate the growth inhibiting and apoptosis inducing properties of SFS in more detail.

Methods Synergy fermentation supernatant (SFS) and feces blank (FB) were characterised for the contents of short chain fatty acids (SCFA) and desoxycholic acid (DCA). Both HT29 and LT97 cells were treated with SFS and FB for 24 h. The effect on metabolic activity was measured by cell titer blue assay whereas the effect on cell growth was measured by DAPI assay. SFS and FB treated cells were challenged with genotoxic compounds namely H_2O_2 and HNE and effect on DNA damage was determined with the Comet assay to determine the possible protective effects of SFS against DNA damage. GST and catalase enzyme activity assays were performed to investigate the possible mechanisms of detoxification. Induction of apoptosis was measured by poly (ADP-ribose) polymerase (PARP) cleavage and Bid cleavage by Western blot and by measuring caspase activity. Real-time RT-PCR and cDNA microarrays were done to determine mRNA expression of genes involved in apoptosis and cell proliferation.

Results Fermentation of Synergy1 increased the yields of total SCFA in SFS in comparison to the control (FB). In SFS, the total amount of SCFA was 127.6 mmol/l whereas only 48.1 mmol/l was detected in FB. Furthermore, the content of DCA was reduced from 12.3 mmol/l in FB to 3.6 mmol/l in SFS. Therefore, the increase in chemoprotective SCFA and the reduced formation of the potentially toxic secondary bile acid DCA, indicate that fermentation products of Synergy1 may be inversely associated in terms of progression of colon cancer.

The growth of both cell lines was efficiently reduced after incubation with SFS in a concentration and time dependant manner. Interestingly, the SFS and SFM were equally effective in mediating growth inhibition, reflecting the potential of SCFA in secondary chemoprevention. Furthermore, the data showed that the growth inhibitory effect of SFS is stronger in adenoma than in carcinoma cells.

Pre-incubation with SFS resulted in significantly reduced levels of H_2O_2-induced DNA strand breaks (39 % in medium control to 22 % in SFS) in HT29 cells ($p \leq 0.01$) whereas this effect was not significant in LT97 cells.

Incubation with SFS resulted in a significant up-regulation of GSTA4 in HT29 tumour cells. The products of this gene are known to protect against oxidative stress and detoxify endogenous aldehydes, quinones, epoxides and hydroperoxides formed as secondary metabolites during oxidative stress and can therefore reduce carcinogenic compounds. Additionally the FB also tended to significantly increase GSTA4 gene expression in HT29 cells, which indicates the presence of other metabolites that may have their origin from faecal samples rather than from fermented Synergy1.

SFS treatment did not significantly influence expression of catalase gene but resulted in a significant induction of catalase activity in HT29 cells. This suggests that catalase activity is not regulated by up-regulation of transcription but by some yet unknown post-transcriptional or post-translational mechanisms.

The Western blot analysis showed that treatment of LT97 cells for 24 h with SFS significantly increased cleavage of PARP when compared to medium control. However, in HT29 cells, 24 h treatment did not lead to an increase of PARP cleavage indicating that HT29 cells were not sensitive towards SFS-induced apoptosis after 24 h of incubation. Furthermore, incubation of LT97 cells for 24 h with synthetic SCFA also resulted in a significant increase in PARP cleavage. However, the levels of cleaved PARP after treatment with SFM were lower in comparison to SFS which points to the role of some additional apoptosis inducing substances in SFS. There was however, no significant change in the level of full length Bid after incubating both cell lines with SFS 10% for 24h.

Further, the activity of caspase-3 was clearly modulated after incubation of both cell lines with SFS pointing towards the efficacy of SFS in inducing apoptosis in both cell

lines. Additionally, incubation of both cell lines with SFM also resulted in a significant increase in the activity of caspase-3.

In LT97 cells a significant down-regulation of mRNA expression of DR4 and Bax (fold change 0.13 and 0.2 respectively) were observed whereas in HT29 cells a significant up-regulation of mRNA expression of DR5 gene (fold change 2.15) and Bax gene (fold change 1.68) and a significant down-regulation of DR4 gene (fold change 0.33) was observed when compared to medium control.

In LT97 cells custom array results indicated that mRNA expression of 37 genes were up-regulated whereas 53 genes were down-regulated on the basis of the evaluation criteria (≥1.5 or ≤ 0.6) in response to treatment with SFS 10 % for 24 h. Among them only 6 were found to be significantly up-regulated and 36 were significantly down-regulated after incubating with SFS 10 % for 24 h. In contrast, in HT29 cells custom array results indicated a significant up-regulation only of 7 genes out of total 97 genes. Among the 28 genes down-regulated by SFS 10 % in HT29 cells 6 genes were modulated significantly.

Conclusions The results of the present study on fermentation supernatant of inulin revealed valuable information about the role of prebiotics in cancer prevention possibly by increase in SCFA production, decrease in toxic secondary bile acids, inhibition of cell growth, induction of apoptosis and reduction of the exposure of genotoxins in human colon tumour cells. Furthermore, the approach to determine the profile of gene expression in human colon cancer cells provides a relevant possibility to identify target genes and agents that could contribute to chemoprotection in colonic mucosa cells.

7 Zusammenfassung

Einleitung Epidemiologische Studien deuten darauf hin, dass eine Diät reich an Fett und rotem Fleisch das Risiko von Darmkrebs erhöhen kann, während eine hohe Aufnahme von Ballaststoffen und komplexen Kohlenhydraten dagegen schützend wirken kann. Eine der potenziell schützenden Gruppen von Nahrungsbestandteilen sind Präbiotika, nicht-verdaulich Nahrungsinhaltsstoffe, die sich positiv auf das Wachstum und / oder die Aktivität von bestimmten positiven Darmbakterien auswirken und gleichzeitig potenzielle Krankheitserreger auf einem niedrigen Niveau halten. Unter den verschiedenen Präbiotika sind Inulin und Oligofructose die am Besten untersuchten. Fruktane des Inulin-Typs sind die natürlichen Bestandteile eines breiten Spektrums von Gemüse und Früchten. Potenzielle Produkte der Fermentation von Inulin sind SCFA und vor allem ein erhöhter relativer Anteil an Butyrat. Diese Fermentationsprodukte zeigten protektive Wirkungen auf unterschiedliche Stadien der Krebsentwicklung, z.B. durch Inhibierung der Zellproliferation und Induktion der Apoptose in Kolon Adenom- und Karzinomzelllinien.

Ziel Das Ziel dieser Studie war die Charakterisierung von Synergy1-Fermentationsüberständen (SFS) hinsichtlich einer Hemmung des Tumor-Zell-Wachstums, Induktion der Apoptose und Modulation von DNA- Schäden. Außerdem die funktionellen Konsequenzen einer Inkubation dieser Überständen wurde in zwei verschiedenen Darmkrebs Zelllinien verglichen nämlich LT97 (präneoplastische Adenom-Zellen) und HT29 (hoch transformierte Adenokarzinom-Zellen), um Aussagen über die Mechanismen der Chemoprävention treffen zu können. Darüber hinaus wurde ein synthetischer Fermentationsüberstand (SFM), der identische Konzentrationen an SCFA und der sekundären Gallensäure Desoxycholsäure (DCA)

enthielt wie der SFS, hergestellt und die Effekte dieses SFM auf Zellwachstum und Apoptose analysiert.

Methoden Die Konzentrationen an SCFA und DCA im SFS und einer Negativkontrolle (Fäzesblank, FB) wurden mittels Gaschromatographie bestimmt. HT29 und LT97-Zellen wurden mit SFS und FB für 24 h inkubiert. Der Einfluss auf die metabolische Aktivität der Zellen wurde mit Hilfe des *cell titer blue assays*, die Modulation des Zellwachstums unter Verwendung des DAPI-assay bestimmt. Mögliche protektive Effekte von SFS und FB vor H_2O_2- und HNE-induzierten DNA-Schäden wurden mittels Comet-Assay ermittelt. Zur Untersuchung von Mechanismen der Entgiftung wurden Enzymaktivitätsassays (GST und Katalase) durchgeführt. Die Induktion der Apoptose wurde durch Detektion von gespaltener Poly (ADP-Ribose) Polymerase (PARP) im Western Blot sowie durch Caspase-Aktivitäts-Experimente analysiert. Der Einfluss auf die Genexpression von Genen der Entgiftung und der apoptotischen Signaltransduktion wurde mit cDNA microarrays und quantitativer *real-time* RT-PCR bestimmt.

Ergebnisse Die Konzentrationen an SCFA waren im SFS im Vergleich zum FB deutlich erhöht In SFS lag die Gesamtmenge an SCFA bei 127,6 mmol/l, während nur 48,1 mmol/l im FB detektiert wurden. Ferner wurde der Gehalt an DCA von 12,3 mmol/l im FB auf 3,6 mmol/ l im SFS reduziert. Die Zunahme chemopräventiver SCFA und die Reduktion karzinogener sekundärer Gallensäuren deuten darauf hin, dass Fermentationsprodukte von Synergy1 die Progression von Kolontumoren einschränken könnten.

Das Wachstum von beiden Zellenlinien wurde nach Inkubation mit SFS in einer konzentrations- und zeitabhängigen Weise reduziert. Da SFM genauso effektive reduzierende Effekte auf das Wachstum zeigte, scheinen vor allem die SCFA wirksame Bestandteile des SFS zu sein. Ferner, haben die Daten gezeigt, dass die

wachstumshemmende Wirkung von SFS in Adenomzellen stärker ausgeprägt ist als in Karzinomzellen.

Eine vorinkubation mit SFS verringerte in HT29 Zellen signifikant($p \leq 0.01$) H_2O_2-induzierte DNA-Strangbrüche (tail intensity: 39% in Mediumkontrolle, 22% in SFS) wohingegen dieser Effekt in LT97-Zellen nicht signifikant war.

Inkubation mit SFS führte zu einer signifikanten Hochregulierung der GSTA4-Expression in HT29-Zellen. Die Produkte dieser Gene dienen bekanntermaßen dem Schutz gegen oxidativen Stress, endogenen Aldehyde, Quinone, Epoxide und Hydroperoxide, und können daher karzinogene Metabolite entgiften. Zusätzlich führte auch der FB zu einer Zunahme der GSTA4 Gen-Expression in HT29 Zellen, was auf das Vorhandensein weiterer aktiver Metabolite hindeuted.

Die Genexpression von Katalase wurde durch SFS nicht reguliert, wohl aber die Enzymaktivität. Dies deutet daraufhin, dass keine transkriptionelle Regulation, sondern noch unbekannte Post-translationale Mechanismen bei der Regulation von Bedeutung sind.

Die Western-Blot-Analysen zeigten, dass eine Behandlung von LT97 Zellen für 24 h mit SFS zu einer signifikanten Spaltung von PARP führten. Im Gegensatz dazu wurde in SFS-inkubierten HT29 Zellen keine PARP-Spaltung nachgewiesen, in diesen Zellen wurde unter diesen Bedingungen keine Apoptose induziert. Eine Inkubation von LT97 Zellen für 24 h mit SFM führte darüberhinaus ebenfalls zu einem signifikanten Anstieg der PARP-Spaltung. Allerdings war die Spaltung von PARP nach der Behandlung mit SFM niedriger im Vergleich zu SFS. Im SFS sind also zusätzliche Apoptose-induzierende Bestandteile enthalten. Die Aktivität der Caspase-3 wurde in beiden Zelllinien durch SFS signifikant erhöht, was darauf hindeutet, dass in beiden Zelllinien Apoptose induziert wurde. Eine, Inkubation der Zellen mit SFM führte ebenfalls zu einer erhöhten Caspase-3 Aktivität. Nach Inkubation beider

Zelllinien mit SFS 10% für 24 Stunden konnte kein Einfluss auf die Bid-Spaltung nachgewiesen werden.

In LT97 Zellen wurde eine signifikante Runterregulierung der mRNA Expression von DR4 (0.13 fach) und Bax (0.2 fach) durch Behandlung mit SFS festgestellt. Im Gegensatz dazu wurde in HT29 Zellen wurde eine signifikante Hochregulierung der mRNA Expression von DR5 (2,15 fach) und Bax (1,68) und eine deutliche Verminderung der Expression von DR4 (0,33-fach) durch Behandlung mit SFS festgestellt.

Die Auswertung der cDNA microarrays zeigte, dass in LT97-Zellen die Expression von 37 Genen gesteigert und von 53 Genen durch die Behandlung mit 10% SFS für 24 h gesenkt wurde (>1,5 bzw. < 0,6). Nur 6 der hochregulierten Gene und 36 der runterregulierten Gene wurden signifikant moduliert. Im Gegensatz dazu wurden in HT29-Zellen 97 Gene hochreguliert (davon 6 signifikant) und 28 Gene herunterreguliert (davon 6 signifikant).

Schlussfolgerung Die Ergebnisse der vorliegenden Studie liefern wertvolle Informationen über die Rolle des Präbiotikums Synergy1 in der Krebs-Prävention z.B. durch Erhöhung der SCFA Produktion, Verringerung der toxischen sekundären Gallensäuren, Hemmung des Wachstums, Induktion der Apoptose und der Verringerung der Exposition mit Genotoxinen in menschlichen Dickdarm-Tumor-Zellen. Weiterhin bietet die Bestimmung des Profils der Genexpression in menschlichen Dickdarm-Krebs-Zellen eine Möglichkeit, relevante Gene bzw. Agenzien zu identifizieren, die dazu beitragen könnten *in vivo* um chemoprotektiv in Dickdarm-Mukosa zu wirken.

8 References

Abdulla M, Gruber P (2000) Role of diet modification in cancer prevention. *Biofactors* **12**: 45-51

Abrahamse SL, Pool-Zobel BL, Rechkemmer G (1999) Potential of short chain fatty acids to modulate the induction of DNA damage and changes in the intracellular calcium concentration by oxidative stress in isolated rat distal colon cells. *Carcinogenesis* **20**: 629-634

Aebi H (1984) Catalase in vitro. *Methods Enzymol* **105**: 121-126

Alberts DS, Einspahr JG, Earnest DL, Krutzsch MF, Lin P, Hess LM, Heddens DK, Roe DJ, Martinez ME, Salen G, Batta AK (2003) Fecal bile acid concentrations in a subpopulation of the wheat bran fiber colon polyp trial. *Cancer Epidemiol Biomarkers Prev* **12**: 197-200

Ashkenazi A, Dixit VM (1998) Death receptors: signaling and modulation. *Science* **281**: 1305-1308

Barry JL, Hoebler C, Macfarlane GT, Macfarlane S, Mathers JC, Reed KA, Mortensen PB, Nordgaard I, Rowland IR, Rumney CJ (1995) Estimation of the fermentability of dietary fibre in vitro: a European interlaboratory study. *Br J Nutr* **74**: 303-322

Bartoli GM, Palozza P, Marra G, Armelao F, Franceschelli P, Luberto C, Sgarlata E, Piccioni E, Anti M (1993) n-3 PUFA and alpha-tocopherol control of tumor cell proliferation. *Mol Aspects Med* **14**: 247-252

Berger A, Roberts MA, Hoff B (2006) How dietary arachidonic- and docosahexaenoic-acid rich oils differentially affect the murine hepatic transcriptome. *Lipids Health Dis* **5**: 10

Berlau J, Glei M, Pool-Zobel BL (2004) Colon cancer risk factors from nutrition. *Anal Bioanal Chem* **378**: 737-743

Bernstein H, Bernstein C, Payne CM, Dvorakova K, Garewal H (2005) Bile acids as carcinogens in human gastrointestinal cancers. *Mutat Res* **589**: 47-65

Beyer-Sehlmeyer G, Glei M, Hartmann E, Hughes R, Persin C, Bohm V, Rowland I, Schubert R, Jahreis G, Pool-Zobel BL (2003) Butyrate is only one of several growth

inhibitors produced during gut flora-mediated fermentation of dietary fibre sources. *Br J Nutr* **90**: 1057-1070

Bingham SA (1996) Epidemiology and mechanisms relating diet to risk of colorectal cancer. *Nutr Res Rev* **9**: 197-239

Bingham SA, Day NE, Luben R, Ferrari P, Slimani N, Norat T, Clavel-Chapelon F, Kesse E, Nieters A, Boeing H, Tjonneland A, Overvad K, Martinez C, Dorronsoro M, Gonzalez CA, Key TJ, Trichopoulou A, Naska A, Vineis P, Tumino R, Krogh V, Bueno-de-Mesquita HB, Peeters PH, Berglund G, Hallmans G, Lund E, Skeie G, Kaaks R, Riboli E (2003) Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet* **361**: 1496-1501

Boutron-Ruault MC, Marteau P, Lavergne-Slove A, Myara A, Gerhardt MF, Franchisseur C, Bornet F (2005) Effects of a 3-mo consumption of short-chain fructo-oligosaccharides on parameters of colorectal carcinogenesis in patients with or without small or large colorectal adenomas. *Nutr Cancer* **53**: 160-168

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254

Bradlow HL, Telang NT, Sepkovic DW, Osborne MP (1999) Phytochemicals as modulators of cancer risk. *Adv Exp Med Biol* **472**: 207-221

Brentnall TA, Crispin DA, Bronner MP, Cherian SP, Hueffed M, Rabinovitch PS, Rubin CE, Haggitt RC, Boland CR (1996) Microsatellite instability in nonneoplastic mucosa from patients with chronic ulcerative colitis. *Cancer Res* **56**: 1237-1240

Brink M, Weijenberg MP, de Goeij AF, Schouten LJ, Koedijk FD, Roemen GM, Lentjes MH, de Bruine AP, Goldbohm RA, van den Brandt PA (2004) Fat and K-ras mutations in sporadic colorectal cancer in The Netherlands Cohort Study. *Carcinogenesis* **25**: 1619-1628

Browne SJ, MacFarlane M, Cohen GM, Paraskeva C (1998) The adenomatous polyposis coli protein and retinoblastoma protein are cleaved early in apoptosis and are potential substrates for caspases. *Cell Death Differ* **5**: 206-213

Buddington KK, Donahoo JB, Buddington RK (2002) Dietary oligofructose and inulin protect mice from enteric and systemic pathogens and tumor inducers. *J Nutr* **132**: 472-477

Burkitt DP (1969) Related disease--related cause? *Lancet* **2**: 1229-1231

Chai F, Evdokiou A, Young GP, Zalewski PD (2000) Involvement of p21(Waf1/Cip1) and its cleavage by DEVD-caspase during apoptosis of colorectal cancer cells induced by butyrate. *Carcinogenesis* **21**: 7-14

Chelikani P, Fita I, Loewen PC (2004) Diversity of structures and properties among catalases. *Cell Mol Life Sci* **61**: 192-208

Clausen MR, Mortensen PB (1995) Kinetic studies on colonocyte metabolism of short chain fatty acids and glucose in ulcerative colitis. *Gut* **37**: 684-689

Cohen E, Ophir I, Shaul YB (1999) Induced differentiation in HT29, a human colon adenocarcinoma cell line. *J Cell Sci* **112 (Pt 16)**: 2657-2666

Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations significant in an initial phase of malignant conversion. **26**: 249-261

Cook SI, Sellin JH (1998) Review article: short chain fatty acids in health and disease. *Aliment Pharmacol Ther* **12**: 499-507

Cory S, Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**: 647-656

Coudray C, Tressol JC, Gueux E, Rayssiguier Y (2003) Effects of inulin-type fructans of different chain length and type of branching on intestinal absorption and balance of calcium and magnesium in rats. *Eur J Nutr* **42**: 91-98

Cuff MA, Lambert DW, Shirazi-Beechey SP (2002) Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. *J Physiol* **539**: 361-371

Cummings JH (1981) Dietary fibre and large bowel cancer. *Proc Nutr Soc* **40**: 7-14

Cummings JH, Englyst HN (1987) Fermentation in the human large intestine and the available substrates. *Am J Clin Nutr* **45**: 1243-1255

Cummings JH, Macfarlane GT (2002) Gastrointestinal effects of prebiotics. *Br J Nutr* **87 Suppl 2**: S145-S151

Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT (1987) Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* **28**: 1221-1227

Daly K, Cuff MA, Fung F, Shirazi-Beechey SP (2005) The importance of colonic butyrate transport to the regulation of genes associated with colonic tissue homoeostasis. *Biochem Soc Trans* **33**: 733-735

de S, Fernando R (1998) Familial adenomatous polyposis. *Ceylon Med J* **43**: 99-105

Degen WG, Pruijn GJ, Raats JM, van Venrooij WJ (2000) Caspase-dependent cleavage of nucleic acids. *Cell Death Differ* **7**: 616-627

Delzenne NM, Daubioul C, Neyrinck A, Lasa M, Taper HS (2002) Inulin and oligofructose modulate lipid metabolism in animals: review of biochemical events and future prospects. *Br J Nutr* **87 Suppl 2**: S255-S259

Desdouets C, Matesic G, Molina CA, Foulkes NS, Sassone-Corsi P, Brechot C, Sobczak-Thepot J (1995) Cell cycle regulation of cyclin A gene expression by the cyclic AMP-responsive transcription factors CREB and CREM. *Mol Cell Biol* **15**: 3301-3309

Desmots F, Rauch C, Henry C, Guillouzo A, Morel F (1998) Genomic organization, 5'-flanking region and chromosomal localization of the human glutathione transferase A4 gene. *Biochem J* **336 (Pt 2)**: 437-442

Dirk Schrijvers HJSHMBZ (2008) *Handbook of Cancer Prevention*. European Society for medical Oncology. Informa Healthcare, UK:

Dove-Edwin I, Thomas HJ (2001) Review article: the prevention of colorectal cancer. *Aliment Pharmacol Ther* **15**: 323-336

Ebert MN, Beyer-Sehlmeyer G, Liegibel UM, Kautenburger T, Becker TW, Pool-Zobel BL (2001) Butyrate induces glutathione S-transferase in human colon cells and protects from genetic damage by 4-hydroxy-2-nonenal. *Nutr Cancer* **41**: 156-164

Enari M, Talanian RV, Wong WW, Nagata S (1996) Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* **380**: 723-726

Femia AP, Luceri C, Dolara P, Giannini A, Biggeri A, Salvadori M, Clune Y, Collins KJ, Paglierani M, Caderni G (2002) Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics Lactobacillus rhamnosus and Bifidobacterium lactis on azoxymethane-induced colon carcinogenesis in rats. *Carcinogenesis* **23**: 1953-1960

Ferguson LR, Harris PJ (2003) The dietary fibre debate: more food for thought. *Lancet* **361**: 1487-1488

Ferguson LR, Tasman-Jones C, Englyst H, Harris PJ (2000) Comparative effects of three resistant starch preparations on transit time and short-chain fatty acid production in rats. *Nutr Cancer* **36**: 230-237

Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. *Mol Aspects Med* **27**: 126-139

Fodde R, Smits R, Clevers H (2001) APC, signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer* **1**: 55-67

Fogh J (1986) Human tumor lines for cancer research. *Cancer Invest* **4**: 157-184

Foulds L (1958) The natural history of cancer. *J Chronic Dis* **8**: 2-37

Fornace AJ, Jr., Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J, Holbrook NJ (1989) Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. *Mol Cell Biol* **9**: 4196-4203

Fuchs CS, Giovannucci EL, Colditz GA, Hunter DJ, Stampfer MJ, Rosner B, Speizer FE, Willett WC (1999) Dietary fiber and the risk of colorectal cancer and adenoma in women. *N Engl J Med* **340**: 169-176

Germain M, Affar EB, D'Amours D, Dixit VM, Salvesen GS, Poirier GG (1999) Cleavage of automodified poly(ADP-ribose) polymerase during apoptosis. Evidence for involvement of caspase-7. *J Biol Chem* **274**: 28379-28384

Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Willett WC (1994) Intake of fat, meat, and fiber in relation to risk of colon cancer in men. *Cancer Res* **54**: 2390-2397

Glei M, Hofmann T, Kuster K, Hollmann J, Lindhauer MG, Pool-Zobel BL (2006) Both wheat (Triticum aestivum) bran arabinoxylans and gut flora-mediated

fermentation products protect human colon cells from genotoxic activities of 4-hydroxynonenal and hydrogen peroxide. *J Agric Food Chem* **54**: 2088-2095

Gwyn K, Sinicrope FA (2002) Chemoprevention of Colorectal Cancer. *American Journal of Gastroenterology* **97** 13-21

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**: 7130-7139

Hague A, Hicks DJ, Hasan F, Smartt H, Cohen GM, Paraskeva C, MacFarlane M (2005) Increased sensitivity to TRAIL-induced apoptosis occurs during the adenoma to carcinoma transition of colorectal carcinogenesis. *Br J Cancer* **92**: 736-742

Hague A, Manning AM, Hanlon KA, Huschtscha LI, Hart D, Paraskeva C (1993) Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int J Cancer* **55**: 498-505

Hague A, Paraskeva C (1995) The short-chain fatty acid butyrate induces apoptosis in colorectal tumour cell lines. *Eur J Cancer Prev* **4**: 359-364

Hall PA, Coates PJ, Ansari B, Hopwood D (1994) Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J Cell Sci* **107 (Pt 12)**: 3569-3577

Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ (2008) Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* **27**: 104-119

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* **100**: 57-70

Harris PJ, Ferguson LR (1993) Dietary fibre: its composition and role in protection against colorectal cancer. *Mutat Res* **290**: 97-110

Harvey KJ, Blomquist JF, Ucker DS (1998) Commitment and effector phases of the physiological cell death pathway elucidated with respect to Bcl-2 caspase, and cyclin-dependent kinase activities. *Mol Cell Biol* **18**: 2912-2922

Hayes JD, Pulford DJ (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* **30**: 445-600

Hernandez A, Thomas R, Smith F, Sandberg J, Kim S, Chung DH, Evers BM (2001) Butyrate sensitizes human colon cancer cells to TRAIL-mediated apoptosis. *Surgery* **130**: 265-272

Hill MJ (1997) Nutrition and human cancer. *Ann N Y Acad Sci* **833**: 68-78

Hill MJ (1995) Bacterial fermentation of complex carbohydrate in the human colon. *Eur J Cancer Prev* **4**: 353-358

Hill MJ, Morson BC, Bussey HJ (1978) Aetiology of adenoma--carcinoma sequence in large bowel. *Lancet* **1**: 245-247

Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991) p53 mutations in human cancers. *Science* **253**: 49-53

Howe GR, Benito E, Castelleto R, Cornee J, Esteve J, Gallagher RP, Iscovich JM, ng-ao J, Kaaks R, Kune GA, . (1992) Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies. *J Natl Cancer Inst* **84**: 1887-1896

Jass JR, Smyrk TC, Stewart SM, Lane MR, Lanspa SJ, Lynch HT (1994) Pathology of hereditary non-polyposis colorectal cancer. *Anticancer Res* **14**: 1631-1634

Johnson IT (1995) Butyrate and markers of neoplastic change in the colon. *Eur J Cancer Prev* **4**: 365-371

Johnson IT, Williamson G, Musk SR (1994) Anticarcinogenic factors in plant foods: a new class of nutrients? *Nutr Res Rev* **7**: 175-204

Jonathan WDV (2003) On Defining dietary fibre. *Proceedings of Nutrition Society* **62**: 37-43

Kanduc D, Capuano F, Capurso SA, Geliebter J, Guercia D, Lucchese A, Mittelman A, Simone SM, Sinha AA, Tiwari R, Farber E (2003) Cancer prevention and therapy: strategies and problems. *J Exp Ther Oncol* **3**: 108-114

Kapiteijn E, Liefers GJ, Los LC, Kranenbarg EK, Hermans J, Tollenaar RA, Moriya Y, van d, V, van Krieken JH (2001) Mechanisms of oncogenesis in colon versus rectal cancer. *J Pathol* **195**: 171-178

Keller S, Jahreis G (2004) Determination of underivatised sterols and bile acid trimethyl silyl ether methyl esters by gas chromatography-mass spectrometry-single ion monitoring in feces. *J Chromatogr B Analyt Technol Biomed Life Sci* **813**: 199-207

Keown-Eyssen GE, Bright-See E (1984) Dietary factors in colon cancer: international relationships. *Nutr Cancer* **6**: 160-170

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**: 239-257

Kiefer J, Beyer-Sehlmeyer G, Pool-Zobel BL (2006) Mixtures of SCFA, composed according to physiologically available concentrations in the gut lumen, modulate histone acetylation in human HT29 colon cancer cells. *Br J Nutr* **96**: 803-810

Kim J, Hong SJ, Lim EK, Yu YS, Kim SW, Roh JH, Do IG, Joh JW, Kim DS (2009) Expression of nicotinamide N-methyltransferase in hepatocellular carcinoma is associated with poor prognosis. *J Exp Clin Cancer Res* **28**: 20

Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. *Cell* **87**: 159-170

Kischkel FC, Lawrence DA, Tinel A, LeBlanc H, Virmani A, Schow P, Gazdar A, Blenis J, Arnott D, Ashkenazi A (2001) Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J Biol Chem* **276**: 46639-46646

Klenow S, Glei M, Haber B, Owen R, Pool-Zobel BL (2008) Carob fibre compounds modulate parameters of cell growth differently in human HT29 colon adenocarcinoma cells than in LT97 colon adenoma cells. *Food Chem Toxicol* **46**: 1389-1397

Klinder A, Forster A, Caderni G, Femia AP, Pool-Zobel BL (2004a) Fecal water genotoxicity is predictive of tumor-preventive activities by inulin-like oligofructoses, probiotics (Lactobacillus rhamnosus and Bifidobacterium lactis), and their synbiotic combination. *Nutr Cancer* **49**: 144-155

Klinder, A., Gietl, E., Hughes, R., Jonkers, N., Karlsson, P., McGlyn, H., Pistoli, S., Tuohy, K., Rafter, J., Rowland, I. R., van Loo, J., and Pool-Zobel, B. L. (2004b) Gut Fermentation Products of Inulin-Derived prebiotics Beneficially Modulate Markers of Tumour Progression in Human Colon Tumour Cells. *International Journal of Cancer Prevention* **1**: 19-32

Knoll N, Ruhe C, Veeriah S, Sauer J, Glei M, Gallagher EP, Pool-Zobel BL (2005) Genotoxicity of 4-hydroxy-2-nonenal in human colon tumor cells is associated with cellular levels of glutathione and the modulation of glutathione S-transferase A4 expression by butyrate. *Toxicol Sci* **86**: 27-35

Knowles LM, Milner JA (2000) Allyl sulfides modify cell growth. *Drug Metabol Drug Interact* **17**: 81-107

Kruh J (1982) Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol Cell Biochem* **42**: 65-82

Kumar S (2007) Caspase fuction in programmed cell death. *Cell Death Differ* **14**: 32-43

Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B, Hillan K, Totpal K, DeForge L, Schow P, Hooley J, Sherwood S, Pai R, Leung S, Khan L, Gliniak B, Bussiere J, Smith CA, Strom SS, Kelley S, Fox JA, Thomas D, Ashkenazi A (2001) Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* **7**: 383-385

Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371**: 346-347

Li H, Zhu H, Xu CJ, Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**: 491-501

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* **94**: 481-490

Lynch HT, de la CA (2003) Hereditary colorectal cancer. *N Engl J Med* **348**: 919-932

Lynch HT, Lynch J (2000) Lynch syndrome: genetics, natural history, genetic counseling, and prevention. *J Clin Oncol* **18**: 19S-31S

Mandal M, Olson DJ, Sharma T, Vadlamudi RK, Kumar R (2001) Butyric acid induces apoptosis by up-regulating Bax expression via stimulation of the c-Jun N-terminal kinase/activation protein-1 pathway in human colon cancer cells. *Gastroenterology* **120**: 71-78

Mandic A, Viktorsson K, Molin M, Akusjarvi G, Eguchi H, Hayashi SI, Toi M, Hansson J, Linder S, Shoshan MC (2001) Cisplatin induces the proapoptotic conformation of Bak in a deltaMEKK1-dependent manner. *Mol Cell Biol* **21**: 3684-3691

Mates JM, Sanchez-Jimenez FM (2000) Role of reactive oxygen species in apoptosis: implications for cancer therapy. *Int J Biochem Cell Biol* **32**: 157-170

Maurer CA, Friess H, Buhler SS, Wahl BR, Graber H, Zimmermann A, Buchler MW (1998) Apoptosis inhibiting factor Bcl-xL might be the crucial member of the Bcl-2 gene family in colorectal cancer. *Dig Dis Sci* **43**: 2641-2648

McEntee MF, Chiu CH, Whelan J (1999) Relationship of beta-catenin and Bcl-2 expression to sulindac-induced regression of intestinal tumors in Min mice. *Carcinogenesis* **20**: 635-640

McMichael AJ, McCall MG, Hartshorne JM, Woodings TL (1980) Patterns of gastro-intestinal cancer in European migrants to Australia: the role of dietary change. *Int J Cancer* **25**: 431-437

McMillan L, Butcher S, Wallis Y, Neoptolemos JP, Lord JM (2000) Bile acids reduce the apoptosis-inducing effects of sodium butyrate on human colon adenoma (AA/C1) cells: implications for colon carcinogenesis. *Biochem Biophys Res Commun* **273**: 45-49

Medina V, Edmonds B, Young GP, James R, Appleton S, Zalewski PD (1997) Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. *Cancer Res* **57**: 3697-3707

Milner JA (2002) Strategies for cancer prevention: the role of diet. *Br J Nutr* **87 Suppl 2**: S265-S272

Milovic V, Teller IC, Faust D, Caspary WF, Stein J (2002) Effects of deoxycholate on human colon cancer cells: apoptosis or proliferation. *Eur J Clin Invest* **32**: 29-34

Moshfegh AJ, Friday JE, Goldman JP, Ahuja JK (1999) Presence of inulin and oligofructose in the diets of Americans. *J Nutr* **129**: 1407S-1411S

Murillo G, Mehta RG (2005) Chemoprevention of chemically-induced mammary and colon carcinogenesis by 1alpha-hydroxyvitamin D5. *J Steroid Biochem Mol Biol* **97**: 129-136

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, . (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**: 37-43

Nordberg M (1998) Metallothioneins: historical review and state of knowledge. *Talanta* **46**: 243-254

Oberreuther-Moschner DL, Rechkemmer G, Pool-Zobel BL (2005) Basal colon crypt cells are more sensitive than surface cells toward hydrogen peroxide, a factor of oxidative stress. *Toxicol Lett* **159**: 212-218

Ogiwara T, Satoh K, Kadoma Y, Murakami Y, Unten S, Atsumi T, Sakagami H, Fujisawa S (2002) Radical scavenging activity and cytotoxicity of ferulic acid. *Anticancer Res* **22**: 2711-2717

Owen RW (1997) Faecal steroids and colorectal carcinogenesis. *Scand J Gastroenterol Suppl* **222**: 76-82

Ozaki Y (1992) Antiinflammatory effect of tetramethylpyrazine and ferulic acid. *Chem Pharm Bull (Tokyo)* **40**: 954-956

Parkin DM, Muir CS (1992) Cancer Incidence in Five Continents. Comparability and quality of data. *IARC Sci Publ* 45-173

Patlolla AK, Barnes C, Yedjou C, Velma VR, Tchounwou PB (2008) Oxidative stress, DNA damage, and antioxidant enzyme activity induced by hexavalent chromium in Sprague-Dawley rats. *Environ Toxicol* **24**: 66-73

Paunesku T, Mittal S, Protic M, Oryhon J, Korolev SV, Joachimiak A, Woloschak GE (2001) Proliferating cell nuclear antigen (PCNA): ringmaster of the genome. *Int J Radiat Biol* **77**: 1007-1021

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45

Phillips J, Muir JG, Birkett A, Lu ZX, Jones GP, O'Dea K, Young GP (1995) Effect of resistant starch on fecal bulk and fermentation-dependent events in humans. *Am J Clin Nutr* **62**: 121-130

Pisani, P , Parkin, D.M., Bray, F. and Ferlay, J. (1999) Estimates of the worldwide mortality from 25 cancers in 1990. *Int. J. Cancer*, **83**: 18-23

Pool-Zobel BL (2005) Inulin-type fructans and reduction in colon cancer risk: review of experimental and human data. *Br J Nutr* **93 Suppl 1**: S73-S90

Pool-Zobel BL, Sauer J (2007) Overview of experimental data on reduction of colorectal cancer risk by inulin-type fructans. *J Nutr* **137**: 2580S-2584S

Pool-Zobel BL, Selvaraju V, Sauer J, Kautenburger T, Kiefer J, Richter KK, Soom M, Wolfl S (2005) Butyrate may enhance toxicological defence in primary, adenoma and tumor human colon cells by favourably modulating expression of glutathione S-transferases genes, an approach in nutrigenomics. *Carcinogenesis* **26**: 1064-1076

Potter JD (1999) Colorectal cancer: molecules and populations. *J Natl Cancer Inst* **91**: 916-932

Poulsen M, Molck AM, Jacobsen BL (2002) Different effects of short- and long-chained fructans on large intestinal physiology and carcinogen-induced aberrant crypt foci in rats. *Nutr Cancer* **42**: 194-205

Rafter J, Bennett M, Caderni G, Clune Y, Hughes R, Karlsson PC, Klinder A, O'Riordan M, O'Sullivan GC, Pool-Zobel B, Rechkemmer G, Roller M, Rowland I, Salvadori M, Thijs H, Van LJ, Watzl B, Collins JK (2007) Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. *Am J Clin Nutr* **85**: 488-496

Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC, Perucho M (1997) Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* **275**: 967-969

Raschke M, Rowland IR, Magee PJ, Pool-Zobel BL (2006) Genistein protects prostate cells against hydrogen peroxide-induced DNA damage and induces expression of genes involved in the defence against oxidative stress. *Carcinogenesis* **27**: 2322-2330

Reddy BS (1999) Possible mechanisms by which pro- and prebiotics influence colon carcinogenesis and tumor growth. *J Nutr* **129**: 1478S-1482S

Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. *Nature* **434**: 843-850

Richter M, Jurek D, Wrba F, Kaserer K, Wurzer G, Karner-Hanusch J, Marian B (2002) Cells obtained from colorectal microadenomas mirror early premalignant growth patterns in vitro. *Eur J Cancer* **38**: 1937-1945

Roberfroid M (1993) Dietary fiber, inulin, and oligofructose: a review comparing their physiological effects. *Crit Rev Food Sci Nutr* **33**: 103-148

Roberfroid MB (2007) Inulin-type fructans: functional food ingredients. *J Nutr* **137**: 2493S-2502S

Roberfroid MB (2005) Introducing inulin-type fructans. *Br J Nutr* **93 Suppl 1**: S13-S25

Rochlitz CF, Herrmann R, de KE (1996) Overexpression and amplification of c-myc during progression of human colorectal cancer. *Oncology* **53**: 448-454

Roessler M, Rollinger W, Palme S, Hagmann ML, Berndt P, Engel AM, Schneidinger B, Pfeffer M, Andres H, Karl J, Bodenmuller H, Ruschoff J, Henkel T, Rohr G, Rossol S, Rosch W, Langen H, Zolg W, Tacke M (2005) Identification of nicotinamide N-methyltransferase as a novel serum tumor marker for colorectal cancer. *Clin Cancer Res* **11**: 6550-6557

Rombeau JL, Kripke SA (1990) Metabolic and intestinal effects of short-chain fatty acids. *JPEN J Parenter Enteral Nutr* **14**: 181S-185S

Rottenberg S, Jaspers JE, Kersbergen A, van der BE, Nygren AO, Zander SA, Derksen PW, de BM, Zevenhoven J, Lau A, Boulter R, Cranston A, O'Connor MJ, Martin NM, Borst P, Jonkers J (2008) High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc Natl Acad Sci U S A* **105**: 17079-17084

Rowland IR, Rumney CJ, Coutts JT, Lievense LC (1998) Effect of Bifidobacterium longum and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. *Carcinogenesis* **19**: 281-285

Ruemmele FM, Dionne S, Qureshi I, Sarma DS, Levy E, Seidman EG (1999) Butyrate mediates Caco-2 cell apoptosis via up-regulation of pro-apoptotic BAK and inducing caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase (PARP). *Cell Death Differ* **6**: 729-735

Ruemmele FM, Schwartz S, Seidman EG, Dionne S, Levy E, Lentze MJ (2003) Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway. *Gut* **52**: 94-100

Ryder K, Lau LF, Nathans D (1988) A gene activated by growth factors is related to the oncogene v-jun. *Proc Natl Acad Sci U S A* **85**: 1487-1491

Sauer J, Richter KK, Pool-Zobel BL (2007b) Products formed during fermentation of the prebiotic inulin with human gut flora enhance expression of biotransformation genes in human primary colon cells. *Br J Nutr* **97**: 928-937

Sauer J, Richter KK, Pool-Zobel BL (2007a) Physiological concentrations of butyrate favorably modulate genes of oxidative and metabolic stress in primary human colon cells. *J Nutr Biochem* **18**: 736-745

Schaeferhenrich A, Beyer-Sehlmeyer G, Festag G, Kuechler A, Haag N, Weise A, Liehr T, Claussen U, Marian B, Sendt W, Scheele J, Pool-Zobel BL (2003) Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal. *Mutat Res* **526**: 19-32

Scheppach W, Bartram HP, Richter F (1995) Role of short-chain fatty acids in the prevention of colorectal cancer. *Eur J Cancer* **31A**: 1077-1080

Schottenfeld D, Beebe-Dimmer JL (2005) Advances in cancer epidemiology: understanding causal mechanisms and the evidence for implementing interventions. *Annu Rev Public Health* **26**: 37-60

Sewing A, Burger C, Brusselbach S, Schalk C, Lucibello FC, Muller R (1993) Human cyclin D1 encodes a labile nuclear protein whose synthesis is directly induced by growth factors and suppressed by cyclic AMP. *J Cell Sci* **104 (Pt 2)**: 545-555

Souleimani A, Asselin C (1993) Regulation of c-myc expression by sodium butyrate in the colon carcinoma cell line Caco-2. *FEBS Lett* **326**: 45-50

Sugio K, Kurata S, Sasaki M, Soejima J, Sasazuki T (1988) Differential expression of c-myc gene and c-fos gene in premalignant and malignant tissues from patients with familial polyposis coli. *Cancer Res* **48**: 4855-4861

Tamakoshi K, Tokudome S, Kuriki K, Takekuma K, Toyoshima H (2001) Epidemiology and primary prevention of colorectal cancer. *Gan To Kagaku Ryoho* **28**: 146-150

Tejpar S, Van CE (2003) Molecular defects in colorectal tumorigenesis. *Drugs Today (Barc)* **39 Suppl C**: 111-118

Ten Bruggencate SJ, Bovee-Oudenhoven IM, Lettink-Wissink ML, Katan MB, van der MR (2004) Dietary fructo-oligosaccharides and inulin decrease resistance of rats to salmonella: protective role of calcium. *Gut* **53**: 530-535

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**: 801-809

Thorburn A (2004) Death receptor-induced cell killing. *Cell Signal* **16**: 139-144

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* **35**: 206-221

Treptow-van LS, Rechkemmer G, Rowland I, Dolara P, Pool-Zobel BL (1999) The carbohydrate crystalean and colonic microflora modulate expression of glutathione S-transferase subunits in colon of rats. *Eur J Nutr* **38**: 76-83

Trock B, Lanza E, Greenwald P (1990) Dietary fiber, vegetables, and colon cancer: critical review and meta-analyses of the epidemiologic evidence. *J Natl Cancer Inst* **82**: 650-661

Trowell H (1976) Definition of dietary fiber and hypotheses that it is a protective factor in certain diseases. *Am J Clin Nutr* **29**: 417-427

Truneh A, Sharma S, Silverman C, Khandekar S, Reddy MP, Deen KC, McLaughlin MM, Srinivasula SM, Livi GP, Marshall LA, Alnemri ES, Williams WV, Doyle ML (2000) Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. *J Biol Chem* **275**: 23319-23325

van Geelen CM, de Vries EG, de JS (2004) Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist Updat* **7**: 345-358

Van Loo J, Clune Y, Bennett M, Collins JK (2005) The SYNCAN project: goals, set-up, first results and settings of the human intervention study. *Br J Nutr* **93 Suppl 1**: S91-S98

Vander Heiden MG, Thompson CB (1999) Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? *Nat Cell Biol* **1**: E209-E216

Velazquez OC, Lederer HM, Rombeau JL (1996) Butyrate and the colonocyte. Implications for neoplasia. *Dig Dis Sci* **41**: 727-739

Verghese M, Rao DR, Chawan CB, Williams LL, Shackelford L (2002) Dietary inulin suppresses azoxymethane-induced aberrant crypt foci and colon tumors at the promotion stage in young Fisher 344 rats. *J Nutr* **132**: 2809-2813

vivi-Green C, Polak-Charcon S, Madar Z, Schwartz B (2000) Apoptosis cascade proteins are regulated in vivo by high intracolonic butyrate concentration: correlation with colon cancer inhibition. *Oncol Res* **12**: 83-95

Vogelstein B, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y, White R (1989) Allelotype of colorectal carcinomas. *Science* **244**: 207-211

Wachtershauser A, Stein J (2000) Rationale for the luminal provision of butyrate in intestinal diseases. *Eur J Nutr* **39**: 164-171

Walczak H, Krammer PH (2000) The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Exp Cell Res* **256**: 58-66

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH (1999) Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* **5**: 157-163

Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ (1996) BID: a novel BH3 domain-only death agonist. *Genes Dev* **10**: 2859-2869

Wattenberg LW (1992) Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res* **52**: 2085s-2091s

WCRF/AICR (2007) *Food, Nutrition, Physical activity, and the Prevention of Cancer: a Global Perspective. Washington DC.*

Weaver CM, Liebman M (2002) Biomarkers of bone health appropriate for evaluating functional foods designed to reduce risk of osteoporosis. *Br J Nutr* **88 Suppl 2**: S225-S232

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**: 727-730

Weitz J, Koch M, Debus J, Hohler T, Galle PR, Buchler MW (2005) Colorectal cancer. *Lancet* **365**: 153-165

Williams EA, Coxhead JM, Mathers JC (2003) Anti-cancer effects of butyrate: use of micro-array technology to investigate mechanisms. *Proc Nutr Soc* **62**: 107-115

Wong JM, de SR, Kendall CW, Emam A, Jenkins DJ (2006) Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol* **40**: 235-243

Yang XH, Sladek TL, Liu X, Butler BR, Froelich CJ, Thor AD (2001) Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis. *Cancer Res* **61**: 348-354

Yi X, Yin XM, Dong Z (2003) Inhibition of Bid-induced apoptosis by Bcl-2. tBid insertion, Bax translocation, and Bax/Bak oligomerization suppressed. *J Biol Chem* **278**: 16992-16999

9 Appendix

Appendix A: List of solutions used during the study

Cell culture

HT29 cell growth medium
450 ml DMEM
50 ml FCS
Stored at 4°C

Trypsin-Versen
10 ml Trypsin [10x] 25g/ml in physiologic saline
100 ml Versen [1x] 0.2g/l in isotonic saline
Stored at 4°C

Phosphate buffer saline (PBS)
8.0 g NaCl
0.2 g/l KCl
0.2 g/l KH_2HPO_4
111.15 g/l $Na_2HPO_4 \times 2\ H_2O$
pH 7.3; autoclaved; Stored at 4°C

PBS + Ca^{2+} + Mg^{2+}
PBS along with addition of:
0.13 g/l $CaCl_2 \times 2\ H_2O$
0.1 g/l $MgCl_2 \times 2\ H_2O$
pH 7.3; sterile filtration; Stored at 4°C

PBS + Ca^{2+} + Mg^{2+} + BSA
PBS + Ca^{2+} + Mg^{2+} along withaddition of
5.0 g/l BSA
pH 7.3; sterile filtration; Stored at 4°C

DAPI-Assay
DAPI-Stem solution
3 mM DAPI
In Methanol

Stored in dark at -20°C

DAPI-working solution
0.02 mM DAPI-Stem solution
in PBS + Ca^{2+} + Mg^{2+}.
Prepared fresh before use

Comet-Assay
Normal Melting Agarose (NMA, 0.5 %)
50 mg NMA
+ 10 ml PBS
Melt it in microwave and keep it in water bath at 60°C

Low Melting Agarose (LMA, 0.7 %)
70 mg LMA
+ 10 ml PBS
Melt it in microwave and keep it in water bath at 40°C

Lysis buffer (stem buffer) (pH 10)

146.1 g/l NaCl (2.5 M)
+ 37.2 g/l Na_2EDTA (100 mM)
+ 1.2 g/l Tris-Base (10 mM)
Adjust pH-value at 10 with approx. 8g 1N NaOH, Add 10g Na-Lauroylsarcosinate (1 %), sterile filtered and kept at room temperature

Lysis buffer (working buffer)

4 ml Triton-X (1 %)
+ 40 ml DMSO (10 %)
+ 356 ml Lysistembuffer (89 %)
Always prepare fresh before use and store it at 4°C

NaOH-Stem solution
400 g/L NaOH (10M)
+ 74.4 g/L Na_2EDTA (200mM)

Elektrophoresis buffer (pH 13)

60 ml NaOH-Stem solution (300mM)
+ 10 l Na_2EDTA-Stem solution (1mM)
+ 1930 ml Milli-Q-Wasser
Always prepare fresh before use and store it at 4°C

Neutralisations buffer (pH 7.5)

Dissolve 48.5 g/l Tris-Base in Milli-Q-Wasser (0.4mM) and adjust pH to 7.5 with concentrated HCl. Autoclave and store it at 4°C

Antifade-Buffer
2.5 g DABCO
+ TAE-Puffer (pH 8, 10 mM Tris-HCl, 1mM EDTA)
50ml Glycerol
Store at 4°C

SYBR Green®-solution

999 µl Antifade-Buffer
+1 µl SYBR Green®-Stem solution
Store it in dark at –20°C

Western Blot

Bradford Reagent

50 mg	Coomassie Brilliant Blue G250 [0.01 %]
23.5 ml	EtOH abs. [0.8 M]
50 ml	Phosphoric acid [1.6 M]
Ad 500 ml	H_2O bidest

Stored in brown bottle at 4°C, for couple of months.

Stem buffer for separating gel (1.5M Tris)

36.6 g	Tris (Base)
Ad 200 ml	H_2O bidest

Adjust pH 8.8 with HCl; Stored at 4°C, 6 weeks

Stem buffer for collecting gel (0.5 M Tris)

3 g	Tris (Base)
Ad 50 ml	H_2O bidest

Adjust pH 6.8 with HCl; Stored at 4°C, 6 weeks

Stem buffer for Electrophoresis and transfer buffer (10 x)

30 g Tris (Base)
144 g Glycine
Ad 1 l H_20 bidest
pH control (pH approx. 8,8); Stored at 4°C, few weeks.

SDS (Sodiumdodecylsulfate) Stem solution (10 %)
25 g SDS
Ad 250 ml H_2O bidest
Stored in dark at 20°C

APS (Ammoniumperoxidisulfate) Stem solution (10 %)
1 g APS
Ad 5 ml H_2O bidest
Stored at -20°C, can be stored for long periods

Bromphenolblue Stem solution (0.6 %)
60 mg Bromphenolblue
Ad 10 ml H_2O bidest
Filtered and kept at 4°C in dark

DTT (Dithiothreithol) Stem solution
3.7 g DTT
Ad 10 ml H_2O bidest
Sterile filtration; Stored at -20°C

Working solution
Separating gel (10 %): Total Volume 15 ml
5.935 ml H_2O bidest
3.750 ml Separating gel –Stem buffer (1.5 M)
5 ml Rotiphorese Gel 30 (30 %)
150 µl SDS Stem solution (10 %)
150 µl APS Stem solution (10 %)
15 µl Tetramethylethylenediamine (TEMED)
Polymerisation time approx. 10-15 min

Collecting gel (3 %): Total Volume 5ml
3.05 ml H_2O bidest
1.2 ml Collecting gel stembuffer (0.5 M)
650 µl Rotiphorese Gel 30 (30 %)

50 µl SDS Stemsolution (10 %)
50 µl APS Stemsolution (10 %)
10 µl TEMED
Polymerisation time approx. 10-15 min

SDS-Page-Loading buffer: Total Volume 10 ml
2.5 ml Collecting gel-Stemsolution (0.5 M)
2 ml SDS Stemsolution (10 %)
1 ml Glycerol (99 %)
3.6 g Urea (6 M)
10 mg Bromphenolblue Stemsolution (0.1 % M)
500 mg DTT Stemsolution (324 mM)
1.5 ml H_2O bidest

Elektrophoresisbuffer: 1-2 Gels
70 ml 10x Elektrophoresis stem buffer
7 ml SDS Stemsolution (10 %)
Add 700 ml H_2O bidest
Always prepare fresh

Transferbuffer (10 %)
200 ml 10 x Elektrophoresistembuffer
200 ml Methanol
Ad 2 l H_2O bidest
Stored at 4°C

10 x TBS (Tris-buffered saline)
60 g Tris base
160 g NaCl
1600 ml H_2O bidest
Adjust pH at 7.4 with HCl
Ad 2 l H_2O bidest
Autoclaved; Stored 4°C

TBS-T (1 x TBS + 0.05 % Tween 20)
100 ml 10 x TBS
0.5 ml Tween 20
Ad 1 l H_2O
Stored at 4°C

Ponceau-Red solution

0.4 g Ponceau-Red
10 ml Acetic acid 5 %
Ad 200 ml H_2O bidest
Stored at room temperature

Blockingbuffer: 5 % Milk powder

2.5 g Milk powder
Ad 50 ml 1 x TBS
Stored at -20°C

2 x Lysebuffer without Nonidet

4 ml Tris/HCl [20 mM]
1.752 g NaCl [150 mM]
20 ml Glycerin [10 %]
0.116 g EDTA [2 mM]
Stored at 4°C. Shortly before use add DTT, Protease- and Phosphatase inhibitors namely
20 µl Nonidet P4 0 1 %
2 µl Pefabloc SC [0.5 mM]
20 µl PMSF [1 mM]
2 µl Pepstatin A [1 µg/ml]
2 µl Leupeptin [1 µg/ml]
20 µl Sodiumorthovanadate [1 mM]
2 µl DTT [1 mM]

Caspase-Activity-Assay

Stem solutions

Hepes Buffer [1 M]

23.83 g Hepes
Adjust pH at 7.4 with KOH
Add 100 ml H_2O bidest
Stored at 4°C

CHAPS [100 mM]

0,615 g CHAPS
Ad 10 ml H_2O bidest
Stored at 4°C

Reactionsbuffer

4 ml	Hepes Stembuffer [1 M]
20 ml	Glycerine 100 %
Ad 100 ml	H_2O bidest

Stored at 4°C

Working solutions

Lysisbuffer: Total 3.5 ml

875 µl	Hepes Stem buffer [1 M]
875 µl	CHAPS Stem buffer [100 mM]
87.5 µl	DTT Stemsolution [1 M]
3.5 µl	Leupeptin [1 mg/ml]
3.5 µl	Pepstatin A [1 mg/ml]
3.5 µl	Pefabloc [100 mg/ml]
35 µl	Sodiumorthovanadate [100 mM]
35 µl	PMSF in Methanol [100 mM]
1582 µl	H_2O bidest

Substrate Reactionsbuffer (25 µM): Total 1.7 ml

6.8 µl	DTT [1 M]
1629.45 µl	Reactions buffer
63.75 µl	Substrate (Caspase 9, 3, 8) [1 mM]

Substrate Reactionsbuffer (0 µM): Total 0.5 ml

2 µl	DTT [1 M]
498 µl	Reactions buffer

Stem solution Inhibitors (1 mM): 1315 ml

1310 µl	PBS
5 µl	Inhibitor (Caspase 9, 3, 8)

Real-time PCR Gel electrophoresis

Tris-Acetate-EDTA-Buffer- (TAE-Buffer-) Stem solution [50 x]

242.2 g/l	Tris-Base
14.6 g/l	EDTA
47.2 ml/l	Acetic acid

pH 8.3; Stored at room temperature

TAE-Buffer working solution

20 ml	TAE-Buffer [50 x]
980 ml	Milli-Q Water

Stored at room temperature

Appendix B List of Primers

Primer	Sequence [5'-…-3']	Firm
GAPDH_F	ACC CAC TCC TCC ACC TTT GAC	MWG-Biotech AG, Ebersbach
GAPDH_R	TCC ACC ACC CTG TTG CTG TAG	MWG-Biotech AG, Ebersbach
DR4_F	TGT CCA CTT TCG TCT CTG AG	MWG-Biotech AG, Ebersbach
DR4_R	ACA GCA TCA GAG TCT CAG TG	MWG-Biotech AG, Ebersbach
DR5_F	CCA CCT GGA CAC CAT ATC TC	MWG-Biotech AG, Ebersbach
DR5_R	TAC AAT CAC CGA CCT TGA CC	MWG-Biotech AG, Ebersbach
Bid_F	GAC ATG GAG AAG GAG AAG AC	MWG-Biotech AG, Ebersbach
Bid_R	TTC ACT CCA TCC CAT TTC TC	MWG-Biotech AG, Ebersbach
GSTA4_F	CCG GAT GGA GTC CGT GAG ATG G'	MWG-Biotech AG, Ebersbach
GST44_R	CCA TGG GCA CTT GTT GGA ACA GC	MWG-Biotech AG, Ebersbach
CAT_F	TGG ACA AGT ACA ATG CTG AG	MWG-Biotech AG, Ebersbach
CAT_R	TTA GGA TGA ACG CTA AG	MWG-Biotech AG, Ebersbach
BaX_F	TCT GAC GGC AAC TTC AAC TG	MWG-Biotech AG, Ebersbach
BaX_R	GGA GGA AGT CCA ATG TCC AG-3	MWG-Biotech AG, Ebersbach

Appendix C Materials used during the study

Name	Firm
CASY®cups	Innovatis AG (CASY®-Technology), Reutlingen
Gloves	Fisher Scientific GmbH, Schwerte
Pipettes (2ml -50ml), sterile	Fisher Scientific GmbH, Schwerte
Falcon-Tubes (15ml & 50ml)	Eppendorff Vertrieb Deutschland, Hamburg
Filterpapers (S & S, GB004)	Whatman, Dassel
iCycler iQ PCR-Plates	Bio-Rad Laboratories GmbH, München
Western blot Combs	Biometra, Göttingen
Mikrotiterplates (6- & 96-Well)	Nunc GmbH & Co. KG, Wiesbaden
Nitrocellulose membrane (0.45 µm pore size)	Whatman, Dassel
Pasteurpipettes	NeoLab, Heidelberg
PCR- tubes (0,2ml & 0,5ml), sterile	Fisher Scientific GmbH, Schwerte

Pipettetips	Fischer Scientific GmbH, Schwerte
Accu-Jet	Brand GmbH und Co., Wertheim
Eppendorff tubes (1,5ml & 2,0ml)	Eppendorff-Netheler-Hinz GmbH, Hamburg
Eppendorff tubes (1,5ml &2,0ml), safe lock	Eppendorff-Netheler-Hinz GmbH, Hamburg
Cell culture (25cm^2 & 75cm^2)	Fisher Scientific GmbH, Schwerte
Cell scrubber	Greiner Bio-One, Frickenhausen
Weighing Balance	Sartorius AG,Göttingen
Elektrophoresis chamber	Biometra, Göttingen
Fluorescencemicroscope Axiolab HBO 50	Carl Zeiss,Jena
Incubator	Forma Scientific, Inc., Marietta, Ohio
Lightmicroscope Axiovert 25	Carl Zeiss,Jena
Multipipette	Eppendorff-Netheler-Hinz GmbH, Hamburg
Thermomixer	Eppendorff-Netheler-Hinz GmbH, Hamburg
Tablecentrifuge Biofuge fresco	Heraeus Instruments GmbH, Hanau
Waterbath	Memert GmbH, Göttingen
Wamplate	Labotech GmbH, Göttingen
Centrifuge	Heraeus Instruments GmbH, Hanau

Appendix D Computer Software used during the study

Name	Firm
Cell Lab Quanta™ SC-MPL 1.0	Beckman Coulter Inc., Fullerton, USA
Graph Pad PRISM™ 4.0 & 5.0	GraphPad Software, San Diego, USA
icycler Software 3.1	Bio-Rad Laboratories, München
Microsoft® Office Paket 2007	Microsoft Corporation, USA
Nanodrop-Software 3.2.0	Peqlab Biotechnologie GmbH, Erlangen
Quantity One™ 4.0.1	Bio-Rad Laboratories, München
XFLUOR™	Tecan AG, Crailsheim
2100 Expert Software	Agilent Technologies, Santa Clara, USA

Comet assay analysis system (Comet Assay II 1.03)	Perceptive instruments

Appendix E.1 Genes upregulated by SFS in HT29 cells

Gene name	Descriptions	Ratio	SD	P value	Sign	
Phase I						
CYBRD1	Cytochrome b reductase 1	2.65	2.01	0.11	ns	↑
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	2.21	0.88	0.17	ns	↑
CYP24A1	Cytochrome P450, family 24, subfamily A,polypeptide 1	2.00	1.02	0.23	ns	↑
CYP2A6	Cytochrome P450, family 2, subfamily A, polypeptide 6	4.97	5.79	0.27	ns	↑
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	3.92	3.55	0.16	ns	↑
CYP2F1	Cytochrome P450, family 2, subfamily F, polypeptide 1	1.65	0.63	0.09	ns	↑
CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	11.35	8.65	0.15	ns	↑
CYP4A11	Cytochrome P450, family 4, subfamily A, polypeptide 11	44.70	69.84	0.14	ns	↑
CYP4F2	Cytochrome P450, subfamily 4 F, polypeptide 3	2.81	1.73	0.29	ns	↑
CYP7A1	Cytochrome P450, family 7, subfamily A, polypeptide 1	1.92	1.17	0.36	ns	↑
POR	NADPH-Cytochrome P450 Reductase	2.23	1.34	0.25	ns	↑
Phase II						
Acetyltransferases						
MYSTA4	Histone Acetyltransferase MYST4	1.80	1.01	0.38	ns	↑
ODP2	Dihydrolipomide Acetyltransferase Pyruvate Dehydrogenase complex	2.15	1.76	0.24	ns	↑
Epoxide Hydrolases						
EPHX1	Epoxide hydrolase 1, microsomal	1.82	0.78	0.40	ns	↑
EPHX2	Epoxide hydrolase 2, cytoplasmic	2.32	0.99	0.10	ns	↑
Glutathione S-Transferases						
GSTA1	Glutathione S-transferase A1	1.55	0.23	0.27	ns	↑
GSTA4	Glutathione S-transferase A4	1.78	0.24	0.31	ns	↑
GSTM1	Glutathione S-transferase M1	2.18	1.37	0.11	ns	↑
GSTM2	Glutathione S-transferase M2 (muscle)	1.84	1.06	0.39	ns	↑
GSTM4	Glutathione S-transferase M4	2.01	0.98	0.43	ns	↑
GSTM5	Glutathione S-transferase M5	3.95	3.52	0.27	ns	↑
GSTT2	Glutathione S-transferase theta 2	4.10	3.69	0.17	ns	↑
Methyltransferases						
HNMT	Histamine Nmethyltransferase	1.79	1.84	0.50	ns	↑
Sulfotransferases						
CHST7	Carbohydrate sulfotransferase 7	14.17	20.31	0.18	ns	↑
CHST8	Carbohydrate sulfotransferase 8	2.05	1.97	0.44	ns	↑
SULT1C1	Sulfotransferase family, cytosolic, 1C, member 1	3.12	2.77	0.04	*	↑
SULT2B1	Sulfotransferase family,cytosolic, 2B, member 1	1.70	0.19	0.28	ns	↑
TPST1	Tyrosylprotein sulfotransferase 1	2.26	2.39	0.44	ns	↑
TPST2	Tyrosylprotein sulfotransferase 2	1.98	1.56	0.72	ns	↑
GAL3ST1	galactosylceramide Sulfotransferase	4.02	4.64	0.24	ns	↑
GN6ST	Carbohydrate sulfotransferase 2	1.95	1.22	0.25	ns	↑
UDP Glycosyltransferases						

UGT1A3	UDP glycosyltransferase 1 family, polypeptide A3	1.62	0.78	0.19	ns	↑
UGT1A4	UDP glycosyltransferase 1 family, polypeptide A4	1.51	0.48	0.46	ns	↑
UGT2B15	UDP glycosyltransferase 2 family, polypeptide B15	1.85	1.19	0.54	ns	↑
UGT2B7	UDP glycosyltransferase 2 family, polypeptide B7	6.06	7.98	0.18	ns	↑
Phase III drug transporters						
Metallothioneins:						
MT2A	Metallothionein 2A	2.56	1.21	0.04	*	↑
P-glycoproteins						
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP)	3.19	2.33	0.26	ns	↑
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP)	2.68	1.72	0.34	ns	↑
Phase II gene regulation pathway						
CCAAT-enhancer-binding protein (C/EBP) mediated regulation						
FOSB	FOSB Protein	1.77	0.97	0.31	ns	↑
E2F2	E2F transcription factor 2	15.48	23.50	0.16	ns	↑
E2F3	E2F transcription factor 3	1.75	0.96	0.42	ns	↑
ERK1	Extracellular signal-regulated kinase 3	1.90	0.17	0.06	ns	↑
K-RAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.69	1.37	0.45	ns	↑
JUND	Transcription Factor JUND	2.64	0.37	0.003	**	↑
Stress and Signal transduction						
Oxidative or Metabolic Stress						
GSR	Glutathione reductase	1.57	0.45	0.23	ns	↑
HMOX1	Heme oxygenase (decycling) 1	2.19	0.61	0.29	ns	↑
NOS2A	nitric oxide synthase 2A (inducible, hepatocytes)	6.11	7.89	0.33	ns	↑
PRDX1	Peroxiredoxin 1	1.72	0.49	0.19	ns	↑
PGH2	Prostaglandin Syththetase 2 precursor (COX-2) (prostaglandin EN	2.27	1.07	0.29	ns	↑
NMOR2	NRH dehydrogenase 2	1.87	0.45	0.14	ns	↑
DNA Damage and Repair						
ATM	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	2.71	2.81	0.68	ns	↑
ERCC4	Excision repair cross-complementing rodent repair deficiency, complementation group 4	1.95	1.14	0.64	ns	↑
Growth Arrest and Senescence						
EGR1	Early growth response 1	1.52	0.77	0.58	ns	↑
GADD45A	Growth arrest and DNA-damage-inducible, alpha	2.03	0.78	0.34	ns	↑
Apoptosis Signaling						
APAF1	apoptotic protease activating factor	4.82	5.37	0.33	ns	↑
CASP8	Caspase 8, apoptosis-related cysteine protease	2.66	1.42	0.40	ns	↑
TNFRSF10D_HUMAN:	TNF Receptor Superfamily Member 10D	1.67	0.91	0.31	ns	↑
PPP1R13B	Apoptosis Stimulating of P53 Protein 1	1.69	0.90	0.34	ns	↑
CASP9	CASP9: CASPASE 9 Precursor	1.76	0.73	0.28	ns	↑
BAK1	BCL-2 Homologus Antagonist killer	1.54	0.71	0.46	ns	↑
Wnt receptor signaling						
FRZB	Frizzled-related protein	2.19	1.20	0.71	ns	↑
WNT5A	Wingless-type MMTV integration site family, member 5A	1.80	0.78	0.31	ns	↑
WNT5B	Wingless-type MMTV integration site family, member 5B	2.24	1.80	0.37	ns	↑

Protein-Tyrosin Phosphatase with potential role in tumorsupressor in colon						
PTPRF	Protein tyrosine phosphatase, receptor type, F	1.51	0.52	0.34	ns	↑
PTPRG	Protein tyrosine phosphatase, receptor type, G	4.01	2.89	0.33	ns	↑
PTPN6	Protein tyrosine phosphatase, non-receptor type 6	2.03	0.56	0.17	ns	↑
Cell Cycle Arrest-Regulation of cell cycle						
CCNA1	cyclin A	1.86	0.36	0.04	*	↑
CDK6	cyclin-dependent kinase 6	6.66	8.87	0.28	ns	↑
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	3.11	1.19	0.12	ns	↑
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.62	0.71	0.38	ns	↑
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	3.03	0.76	0.03	*	↑
CDKN2D	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	2.84	2.00	0.13	ns	↑
CGRRF1	Cell growth regulator with ring finger domain 1	1.83	1.17	0.19	ns	↑
TGFA	Transforming growth factor, alpha	1.57	0.60	0.23	ns	↑
TP53I3	Tumor Protein 53 Inducible protein P53	2.39	2.89	0.81	ns	↑
PIK3R3	Phosphytidyl inositol 3-Kinase regulatory alpha subunit	1.55	0.44	0.48	ns	↑
CDK1	Cell Division Contriol Protein 2	1.76	1.39	0.51	ns	↑
Metal storage and utilization						
FECH	Ferrochelatase (protoporphyria)	2.32	1.62	0.37	ns	↑
FTH1	Ferritin, heavy polypeptide 1	1.58	0.36	0.18	ns	↑
FTL	Ferritin, light polypeptide	2.21	0.24	0.02	*	↑
SLC11A1	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	2.63	2.15	0.10	ns	↑
SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	1.58	1.35	0.88	ns	↑
TF	Transferrin	1.94	1.48	0.33	ns	↑
HFE_2	Heredirtary Hemochromatiosis Protein Precursor	7.90	10.67	0.20	ns	↑
HFE_3	Heredirtary Hemochromatiosis Protein Precursor.	1.73	0.79	0.24	ns	↑
HFE_4	HFE_4: Heredirtary Hemochromatiosis Protein Precursor	2.82	2.32	0.12	ns	↑
Miscellaneous (several functions)						
AHR	Arylhydrocarbon	2.27	0.89	0.27	ns	↑
ARNT	aryl hydrocarbon receptor nuclear translocator	2.59	1.52	0.05	*	↑
ATF2	activating transcription factor 2	2.25	1.67	0.38	ns	↑
ESR1	estrogen receptor 1 (ER alpha)	2.71	2.35	0.43	ns	↑
ESR2	estrogen receptor 2 (ER beta)	2.22	1.23	0.13	ns	↑
TRAF1	TNF receptor-associated factor 1	2.36	2.54	0.38	ns	↑
UBE2D1	Ubiquitin-conjugating enzyme E2D 1 (UBC4/5 homolog, yeast)	2.32	2.17	0.78	ns	↑
XDH	xanthine dehydrogenase	2.10	1.58	0.65	ns	↑
IAP1	Baculoviral IAP Repeat Containing Protein 3 (inhibitor of Apoptosis)	1.91	0.41	0.31	ns	↑
BIRC6	Baculoviral IAP Repeat Containing Protein 6 (inhibitor of Apoptosis)	5.20	4.07	0.26	ns	↑
BIRC7	Baculoviral IAP Repeat Containing Protein 7 (inhibitor of Apoptosis)	4.90	3.88	0.05	ns	↑

Appendix E.2 Genes down-regulated by SFS in HT 29 cells

Gene name	Descriptions	Ratio	SD	Pvalue	Sign	
Phase I						
CYP11A	Cytochrome P450, family 11, subfamily A, polypeptide 1	0.56	0.13	0.06	ns	↓
Phase II						
Acetyltransferases:						
ACAT2	Acetyl-Coenzyme A acetyltransferase 2	0.30	0.08	0.09	ns	↓
Glutathione S-Transferases						
GSTO1	Glutathione S-transferase omega 1	0.44	0.19	0.07	ns	↓
MGST1	Microsomal glutathione S-transferase1	0.28	0.08	0.08	ns	↓
MGST2	Microsomal glutathione S-transferase 2	0.42	0.08	0.22	ns	↓
Methyltransferases						
COMT	Catechol-Omethyltransferase	0.45	0.18	0.25	ns	↓
NNMT	Nicotinamide Nmethyltransferase	0.58	0.18	0.02	*	↓
Sulfotransferases						
CHST10	Carbohydrate Sulfotransferase 10	0.55	0.18	0.11	ns	↓
UDP Glycosyltransferases						
UGT1A7	UDP glycosyltransferase 1 family, polypeptide A7	0.52	0.04	0.43	ns	↓
Stress and Signal transduction						
Oxidative or Metabolic Stress						
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	0.52	0.18	0.16	ns	↓
GPX1	Glutathione peroxidase 1	0.27	0.09	0.08	ns	↓
HSPD1	Heat shock 60kDa protein 1 (chaperonin)	0.37	0.20	0.13	ns	↓
DNA Damage and Repair						
ERCC6	Excision repair cross-complementing rodent repair deficiency	0.43	0.06	0.04	*	↓
UNG	Uracil-DNA glycosylase	0.30	0.09	0.07	ns	↓
XRCC5	X-ray repair complementing defective repair	0.48	0.22	0.30	ns	↓
Growth Arrest and Senescence						
PCNA	Proliferating cell nuclear antigen	0.26	0.14	0.0036	**	↓
Apoptosis Signaling						
BID	BH3 Interacting Domain Death Agonist	0.45	0.04	0.18	ns	↓
Cell Cycle Arrest-Regulation of cell cycle						
CCNB1	cyclin B1	0.55	0.24	0.43	ns	↓
CDK4	cyclin-dependent kinase 4	0.58	0.18	0.12	ns	↓
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	0.42	0.08	0.18	ns	↓
Metal storage and utilization						
HFE	Hemochromatosis	0.54	0.29	0.04	*	↓
TFRC	Transferrin receptor (p90, CD71)	0.59	0.15	0.54	ns	↓

Miscellaneous (several functions)						
BRCA1	breast cancer 1, early onset	0.50	0.24	0.06	ns	↓
MCM4	MCM4 minichromosome maintenance deficient 4	0.37	0.08	0.05	ns	↓
MCM7	MCM7 minichromosome maintenance deficient	0.39	0.24	0.02	*	↓
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	0.35	0.04	0.05	*	↓
TYMS	Thymidylate synthetase	0.06	0.01	0.06	ns	↓
BIRC5	Baculoviral IAP Repeat Containing Protein 6 (inhibitor of Apoptosis)	0.59	0.04	0.37	ns	↓

Appendix E.3 Genes up-regulated by FB in HT 29 cells

Gene name	Descriptions	Ratio	SD	Pvalue	Sign	
Phase I						
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	1.82	0.61	0.61	ns	↑
CYP2A6	Cytochrome P450, family 2, subfamily A, polypeptide 6	2.76	2.58	0.85	ns	↑
CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6	8.43	9.53	0.81	ns	↑
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6	7.90	9.09	0.91	ns	↑
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	20.14	26.03	0.70	ns	↑
CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	5.39	4.54	0.78	ns	↑
CYP4F2	Cytochrome P450, subfamily 4 F, polypeptide 3	3.21	3.06	0.76	ns	↑
CYP7A1	Cytochrome P450, family 7, subfamily A, polypeptide 1	2.08	1.20	0.80	ns	↑
Phase II						
PIK3CG	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	7.10	8.29	0.88	ns	↑
Glutathione S-Transferases						
GSTM4	Glutathione S-transferase M4	1.66	0.59	0.82	ns	↑
GSTM5	Glutathione S-transferase M5	1.69	0.88	0.90	ns	↑
Methyltransferases						
HNMT	Histamine Nmethyltransferase	1.62	0.95	0.91	ns	↑
Sulfotransferases						
CHST7	Carbohydrate sulfotransferase 7	2.09	0.93	0.96	ns	↑
CHST8	Carbohydrate sulfotransferase 8	1.52	0.82	0.94	ns	↑
SULT1B1	Thyroid hormone slfotransferase	18.34	20.57	0.76	ns	↑
SULT2B1	Sulfotransferase family,cytosolic, 2B, member 1	1.51	0.27	0.52	ns	↑
UDP Glycosyltransferases						
UGT1A6	UDP glycosyltransferase 1 family, polypeptide A6	1.91	0.80	0.66	ns	↑
UGT2B15	UDP glycosyltransferase 2 family, polypeptide B15	2.63	2.19	0.96	ns	↑
P-glycoproteins						
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	1.53	0.71	0.83	ns	↑
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	45.72	62.87	0.98	ns	↑
NF-E2 gene family NRF1 and 2 mediated regulations						
E2F2	E2F transcription factor 2	10.15	12.10	0.94	ns	↑
ERK1	Extracellular signal-regulated kinase 3	1.57	0.29	0.61	ns	↑

Stress and Signal transduction						
Oxidative or Metabolic Stress:						
FMO1	Flavin containing monooxygenase 1	2.89	0.93	0.59	ns	↑
NOS2A	nitric oxide synthase 2A (inducible, hepatocytes)	5.50	3.52	0.77	ns	↑
Growth Arrest and Senescence						
GADD45B	Growth arrest and DNA-damage-inducible, beta	2.03	0.90	0.74	ns	↑
Apoptosis Signaling						
APAF1	apoptotic protease activating factor	2.97	2.70	0.85	ns	↑
CASP8	Caspase 8, apoptosis-related cysteine protease	1.79	1.07	1.00	ns	↑
TNFSF6	Tumor necrosis factor (ligand) superfamily, member 6	1.58	0.85	0.86	ns	↑
Wnt receptor signaling						
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	2.22	1.01	0.96	ns	↑
WNT5B	Wingless-type MMTV integration site family, member 5B	1.51	0.50	0.81	ns	↑
Cell Cycle Arrest-Regulation of cell cycle						
CCNA1	cyclin A	21.94	28.89	0.95	ns	↑
CDK6	cyclin-dependent kinase 6	1.53	0.82	0.99	ns	↑
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.80	0.88	0.99	ns	↑
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p19, inhibits CDK4)	2.57	1.66	1.00	ns	↑
Metal storage and utilization						
FECH	Ferrochelatase (protoporphyria)	1.86	0.90	0.85	ns	↑
SLC11A2	Solute carrier family 11	5.97	6.60	0.82	ns	↑
HFE_2	Heredirtary Hemochromatiosis Protein Precursor	1.85	0.52	0.76	ns	↑
Miscellaneous (several functions)						
AHR	Arylhydrocarbon	1.69	0.46	0.85	ns	↑
ARNT	aryl hydrocarbon receptor nuclear translocator	2.53	2.15	0.96	ns	↑
ATF2	activating transcription factor 2	2.27	1.49	1.00	ns	↑
ESR1	estrogen receptor 1 (ER alpha)	7.95	8.63	0.78	ns	↑
TRAF1	TNF receptor-associated factor 1	2.17	1.13	0.93	ns	↑
BIRC4	Baculoviral IAP Repeat Containing Protein 4 (inhibitor of Apoptosis)	2.59	2.85	0.53	ns	↑
BIRC6	Baculoviral IAP Repeat Containing Protein 6 (inhibitor of Apoptosis)	1.64	0.88	0.88	ns	↑
BIRC7	Baculoviral IAP Repeat Containing Protein 7 (inhibitor of Apoptosis)	2.46	1.59	0.98	ns	↑

Appendix E.4: Genes down-regulated by FB in HT 29 cells

Gene name	Descriptions	Ratio	SD	Pvalue	Sign	
Glutathione S-Transferases						
MGST2	Microsomal glutathione S-transferase 2	0.60	0.18	0.11	ns	↓
Stress and Signal transduction						
Oxidative or Metabolic Stress:						
GPX1	Glutathione peroxidase 1	0.52	0.13	0.36	ns	↓

DNA Damage and Repair						
UNG	Uracil-DNA glycosylase	0.57	0.07	0.04	*	↓
Miscellaneous (several functions)						
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.60	0.03	0.19	ns	↓
TYMS	Thymidylate synthetase	0.50	0.15	0.41	ns	↓

Appendix E.5 Genes up-regulated by SFS in LT 97 cells

Gene name	Descriptions	Ratio	SD	Pvalue	Sign	
Phase I P450 gene family						
CYP2A6	Cytochrome P450, family 2, subfamily A, polypeptide 6	2.38	1.46	0.38	ns	↑
CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	2.42	2.44	0.32	ns	↑
Glutathione S-Transferases						
GSTM3	Glutathione S-transferase M3 (brain)	1.89	0.60	0.25	ns	↑
Sulfotransferases						
SULT1B1	Thyroid hormone slfotransferase	3.26	3.56	0.24	ns	↑
UDP Glycosyltransferases						
UGT2B15	UDP glycosyltransferase 2 family, polypeptide B15	1.52	0.94	0.33	ns	↑
UGT2B4	UDP glycosyltransferase 2 family, polypeptide B4	4.40	3.37	0.14	ns	↑
Phase III drug transporters						
Metallothioneins						
MT2A	Metallothionein 2A	6.11	2.74	0.003	**	↑
P-glycoproteins						
ABCB4	ATP-binding cassette, sub-family B (MDR/TAP)	2.20	1.28	0.21	ns	↑
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP)	2.17	1.14	0.07	ns	↑
NF-E2 gene family NRF1 and 2 mediated regulations						
FOS_2	c-Fos proto-oncogene	7.92	4.37	0.03	*	↑
E2F2	E2F transcription factor 2	1.85	0.97	0.31	ns	↑
Stress and Signal transduction						
Oxidative or Metabolic Stress						
FMO1	Flavin containing monooxygenase 1	2.70	2.95	0.43	ns	↑
HSPA5	Heat shock 70kDa protein 5	3.80	1.32	0.02	*	↑
NOS2A	nitric oxide synthase 2A (inducible, hepatocytes)	1.52	0.19	0.65	ns	↑
PRDX1	Peroxiredoxin 1	1.61	0.78	0.21	ns	↑
DNA Damage and Repair						
ATM	Ataxia telangiectasia mutated	1.70	1.16	0.35	ns	↑
Growth Arrest and Senescence						
DDIT3	DNA-damage-inducible transcript 3	2.21	0.47	0.02	*	↑

GADD45A	Growth arrest and DNA-damage-inducible, alpha	1.96	0.63	0.07	ns	
GADD45B	Growth arrest and DNA-damage-inducible, beta	3.47	2.57	0.16	ns	↑
Apoptosis Signaling						
APAF1	apoptotic protease activating factor	1.92	1.08	0.09	ns	↑
BAX	BCL2-associated X protein	1.53	0.62	0.34	ns	↑
CASP8	Caspase 8, apoptosis-related cysteine protease	3.76	2.72	0.14	ns	↑
Protein-Tyrosin Phosphatase with potential role in tumorsupressor in colon						
PTPN14	Protein tyrosine phosphatase, non-receptor type 14	4.34	3.22	0.08	ns	↑
Cell Cycle Arrest-Regulation of cell cycle:						
CDK6	cyclin-dependent kinase 6	1.70	0.67	0.33	ns	↑
CDKN2B	Cyclin-dependent kinase inhibitor 2B	2.08	0.55	0.15	ns	↑
CDKN2D	Cyclin-dependent kinase inhibitor 2D	2.58	0.33	0.18	ns	↑
CGRRF1	Cell growth regulator with ring finger domain 1	1.74	1.66	0.74	ns	↑
PIK3R1	Phosphatidylinositol 3-Kinase	1.57	0.48	0.42	ns	↑
Metal storage and utilization						
EPO	erythropoietin	2.19	1.00	0.16	ns	↑
HFE	Hemochromatosis	1.72	1.26	0.31	ns	↑
Miscellaneous (several functions)						
ALAS1	Aminolevulinate, delta-, synthase 1	1.67	0.42	0.21	ns	↑
ARNT	aryl hydrocarbon receptor nuclear translocator	1.77	0.60	0.02	*	↑
ATF2	activating transcription factor 2	2.81	1.54	0.11	ns	↑
TRAF1	TNF receptor-associated factor 1	3.66	4.29	0.26	ns	↑
IAP1	Baculoviral IAP Repeast-Containing Protein 3	1.85	0.67	0.11	ns	↑
BIRC4	Baculoviral IAP Repeast-Containing Protein 4	3.15	1.53	0.03	*	↑
BIRC6	Baculoviral IAP Repeat-Containing Protein 6	1.91	0.91	0.21	ns	↑

Appendix E.6 Genes down-regulated by SFS in LT 97 cells

Gene name	Descriptions	Ratio	SD	Pvalue	Sign	
Phase I P450 gene family						
CYP11A	Cytochrome P450	0.59	0.20	0.08	ns	↓
CYP2E1	Cytochrome P450	0.46	0.19	0.08	ns	↓
Phase II						
Acetyltransferases						
ACAT2	Acetyl-Coenzyme A acetyltransferase 2	0.16	0.07	0.0007	***	↓
Epoxide Hydrolases						
EPHX2	Epoxide hydrolase 2, cytoplasmic	0.25	0.06	0.0002	***	↓
Glutathione S-Transferases						
GSTO1	Glutathione S-transferase omega 1	0.42	0.14	0.0019	**	↓
GSTP1	Glutathione S-transferase pi	0.37	0.16	0.17	ns	↓
MGST1	Microsomal glutathione S-transferase1	0.27	0.11	0.01	*	↓
MGST2	Microsomal glutathione S-transferase 2	0.15	0.06	0.0015	**	↓

Methyltransferases						
COMT	Catechol-Omethyltransferase	0.22	0.08	0.011	**	↓
Sulfotransferases						
CHST4	Carbohydrate sulfotransferase 4	0.53	0.13	0.08	ns	↓
SULT1A1	Sulfotransferase family, cytosolic, 1A	0.50	0.17	0.01	*	↓
UDP Glycosyltransferases						
UGT1A7	UDP glycosyltransferase 1 family	0.49	0.18	0.03	*	↓
UGT2B17	UDP glycosyltransferase 2 family	0.57	0.18	0.02	*	↓
P-glycoproteins						
ABCC3	ATP-binding cassette, sub-family C	0.42	0.30	0.09	ns	↓
NF-E2 gene family NRF1 and 2 mediated regulations						
ERK1	Extracellular signal-regulated kinase 3	0.49	0.20	0.16	ns	↓
KEAP1	Kelch-like ECH-associated protein 1	0.50	0.17	0.01	**	↓
NRF2	Nuclear factor (erythroid-derived 2)-like 2	0.39	0.14	0.0047	**	↓
Stress and Signal transduction						
Oxidative or Metabolic Stress						
FMO5	Flavin containing monooxygenase 5	0.56	0.16	0.02	*	↓
GPX1	Glutathione peroxidase 1	0.46	0.15	0.02	*	↓
GPX2	Glutathione peroxidase 2	0.18	0.09	0.02	*	↓
HMOX1	Heme oxygenase (decycling) 1	0.48	0.24	0.05	*	↓
HSPA1A	Heat shock 70kDa protein 1A	3.80	1.32	0.02	*	↓
PRDX2	Peroxiredoxin 2	0.43	0.10	0.04	*	↓
DIA4	NAD(P)H Dehydrogenase	0.60	0.24	0.04	*	↓
DNA Damage and Repair						
RAD51	RAD51 homolog	0.58	0.20	0.06	ns	↓
XRCC2	X-ray repair complementing defective repair	0.43	0.08	0.0021	**	↓
XRCC5	X-ray repair complementing defective repair	0.47	0.14	0.01	*	↓
Growth Arrest and Senescence						
CCND1 (cyclin D1)	Cyclin D1	0.45	0.32	0.04	*	↓
IGFBP6	Insulin-like growth factor binding protein 6	0.54	0.31	0.08	ns	↓
PCNA	Proliferating cell nuclear antigen	0.32	0.17	0.01	**	↓
PAG608: (PAG608)	P53 Target Zinc Finger Protein	0.57	0.20	0.10	ns	↓
Apoptosis Signaling						
BCL2L1 (bcl-x)	BCL2-like 1	0.48	0.28	0.05	ns	↓
CASP3	Caspase 3	0.59	0.17	0.02	*	↓
TNFRSF1A	Tumor necrosis factor receptor superfamily	0.54	0.31	0.06	ns	↓
TNFRSF6 (Fas)	Fas (TNF receptor superfamily, member 6)	0.44	0.18	0.02	*	↓
TNFSF10 (TRAIL)	Tumor necrosis factor (ligand) superfamily	0.58	0.13	0.03	*	↓
PPP1R13B	Apoptosis -Stimulating Of P53 Protein 1	0.60	0.26	0.06	ns	↓
CASP6	CASPASE-6 Precursor	0.34	0.12	0.01	**	↓
BID: (BID)	BH3 interacting domain death agonist BID	0.50	0.13	0.03	*	↓
Cell Cycle Arrest-Regulation of cell cycle						
CCNB1	cyclin B1	0.40	0.23	0.03	*	↓
CCNB2	cyclin B2	0.53	0.23	0.02	*	↓

CDK4	cyclin-dependent kinase 4	0.52	0.28	0.07	ns	↓
PIG3	tumor protein p53 inducible protein 3	0.35	0.17	0.04	*	↓
TP53	Tumor protein p53 (Li-Fraumeni syndrome)	0.57	0.25	0.06	ns	↓
VEGF	Vascular endothelial growth factor	0.54	0.22	0.12	ns	↓
Metal storage and utilization						
FTL	Ferritin, light polypeptide	0.60	0.25	0.05	*	↓
SLC11A2 (DMT1)	Solute carrier family 11	0.31	0.18	0.05	*	↓
Miscellaneous (several functions)						
GSS	Glutathione synthetase	0.41	0.10	0.002	**	↓
MCM7	MCM7 minichromosome maintenance deficient 7	0.23	0.12	0.01	**	↓
MSH2	mutS homolog 2, colon cancer, nonpolyposis	0.49	0.15	0.23	ns	↓
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer	0.31	0.17	0.04	*	↓
PUMA	BCL2 binding component 3	0.54	0.30	0.12	ns	↓
TYMS	Thymidylate synthetase	0.28	0.24	0.03	*	↓

Appendix E.7 Genes up-regulated by FB in LT 97 cells

Gene name	Descriptions	Ratio	SD	Pvalue	Sign	
Phase I						
CYBRD1	Cytochrome b reductase 1	1.66	1.11	0.51	ns	↑
CYP1A1	Cytochrome P450, family 1	2.02	1.23	0.16	ns	↑
CYP24A1	Cytochrome P450, family 1	1.71	1.26	0.48	ns	↑
CYP2A6	Cytochrome P450, family 2	2.86	2.79	0.33	ns	↑
CYP2B6	Cytochrome P450, family 2	2.60	2.29	0.34	ns	↑
CYP2D6	Cytochrome P450, family 2	2.20	1.39	0.39	ns	↑
CYP3A7	Cytochrome P450, family 3	4.34	2.07	0.26	ns	↑
CYP4B1	Cytochrome P450, family 4	1.78	1.11	0.45	ns	↑
CYP4F2	Cytochrome P450, subfamily 4	1.64	0.99	0.38	ns	↑
CYP7A1	Cytochrome P450, family 7	2.21	1.11	0.34	ns	↑
Phase II						
Acetyltransferases						
NAT1	N-acetyltransferase 1	1.65	1.40	0.54	ns	↑
Glutathione S-Transferases						
GSTA1	Glutathione S-transferase A1	1.55	1.08	0.51	ns	↑
GSTA4	Glutathione S-transferase A4	1.53	1.05	0.53	ns	↑
GSTM3	Glutathione S-transferase M3	7.25	5.21	0.29	ns	↑
GSTM5	Glutathione S-transferase M5	1.76	1.07	0.40	ns	↑
GSTP1	Glutathione S-transferase pi	1.59	1.63	0.71	ns	↑
GSTT2	Glutathione S-transferase theta 2	2.19	1.88	0.41	ns	↑
MGST3	Microsomal glutathione S-transferase	1.63	1.08	0.50	ns	↑
Methyltransferases						
HNMT	Histamine Nmethyltransferase	1.64	0.75	0.43	ns	↑
TPMT	Thiopurine Smethyltransferase	1.97	1.32	0.29	ns	↑
Sulfotransferases						
CHST3	Carbohydrate sulfotransferase 3	1.71	1.15	0.57	ns	↑

CHST6	Carbohydrate sulfotransferase 6	2.06	1.68	0.45	ns	↑
CHST7	Carbohydrate sulfotransferase 7	2.62	1.47	0.33	ns	↑
CHST8	Carbohydrate sulfotransferase 8	2.58	1.33	0.30	ns	↑
SULT1B1	Thyroid hormone slfotransferase	3.01	1.31	0.26	ns	↑
SULT2B1	Sulfotransferase family,cytosolic, 2B	1.76	1.23	0.43	ns	↑
TPST1	Tyrosylprotein sulfotransferase 1	1.57	1.15	0.60	ns	↑
TPST2	Tyrosylprotein sulfotransferase 2	1.87	1.42	0.42	ns	↑
GAL3ST1	Galatosylceramide Sulfotransferase	1.53	0.79	0.68	ns	↑
UDP Glycosyltransferases						
UGT1A4	UDP glycosyltransferase 1 family	1.53	1.08	0.52	ns	↑
UGT1A6	UDP glycosyltransferase 1 family	1.80	1.48	0.60	ns	↑
Phase III drug transporters						
Metallothioneins:						
MT2A	Metallothionein 2A	6.12	6.65	0.26	ns	↑
MT3	Metallothionein 3	1.60	1.18	0.54	ns	↑
P-glycoproteins						
ABCB1	ATP-binding cassette, sub-family B	2.67	2.32	0.36	ns	↑
ABCC1	ATP-binding cassette, sub-family C	1.88	1.10	0.47	ns	↑
ABCC2	ATP-binding cassette, sub-family C	5.52	4.89	0.16	ns	↑
ABCG2	ATP-binding cassette, sub-family G	1.81	1.36	0.43	ns	↑
Phase II gene regulation pathway						
CCAAT-enhancer-binding protein (C/EBP) mediated regulation						
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	2.04	1.65	0.42	ns	↑
NF-E2 gene family NRF1 and 2 mediated regulations						
FOS_2	c-Fos proto-oncogene	3.52	3.30	0.25	ns	↑
FOSB	FOSB PROTEIN	1.75	1.27	0.49	ns	↑
E2F2	E2F transcription factor 2	4.33	1.69	0.18	ns	↑
K-RAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.50	1.13	0.88	ns	↑
JUND	JUND Transcriütion Factor JUN-D.	2.05	1.74	0.50	ns	↑
Stress and Signal transduction						
Oxidative or Metabolic Stress						
DNAJA1	DNAJ (Hsp40) homolog, subfamily A	1.59	1.23	0.56	ns	↑
FMO1	Flavin containing monooxygenase 1	2.75	1.73	0.37	ns	↑
HMOX1	Heme oxygenase (decycling) 1	1.72	1.23	0.87	ns	↑
NOS2A	nitric oxide synthase 2A	1.92	1.31	0.25	ns	↑
PRDX1	Peroxiredoxin 1	2.72	2.14	0.31	ns	↑
SOD2	Superoxide dismutase 2, mitochondrial	1.64	1.25	0.56	ns	↑
PGH2	Superoxide dismutase 2, mitochondrial	1.89	1.27	0.46	ns	↑
NMOR2	Prostaglandin Syththetase 2 precursor	1.80	1.38	0.48	ns	↑
HSP90AA1	NRH dehydrogenase 2	1.74	1.40	0.52	ns	↑
DNA Damage and Repair						
ATM	Ataxia telangiectasia mutated	2.99	1.99	0.12	ns	↑
ERCC4	Excision repair cross-complementing	1.51	1.18	0.62	ns	↑
RAD17	RAD17 homolog (S. pombe)	1.52	1.00	0.54	ns	↑
RAD9	RAD9 homolog A (S. pombe)	1.93	1.62	0.62	ns	↑

XRCC3	X-ray repair complementing defective repair	1.84	1.19	0.43	ns	↑
Growth Arrest and Senescence						
DDIT3	DNA-damage-inducible transcript 3	1.56	1.09	0.52	ns	↑
E2F1	E2F transcription factor 1	1.57	1.13	0.52	ns	↑
GADD45B	Growth arrest and DNA-damage	1.64	0.94	0.45	ns	↑
Apoptosis Signaling						
APAF1	apoptotic protease activating factor	3.87	3.01	0.26	ns	↑
BAX	BCL2-associated X protein	4.32	2.38	0.18	ns	↑
TNFRSF10B	tumor necrosis factor receptor	1.59	1.15	0.53	ns	↑
TNFRSF1A	Tumor necrosis factor receptor	1.51	1.23	0.84	ns	↑
TNFSF6	Tumor necrosis factor	2.15	1.76	0.38	ns	↑
CASP9	CASP9: CASPASE 9 Precursor	1.75	1.45	0.62	ns	↑
BAK1	BCL-2 Homologus Antagonist killer	1.58	1.06	0.63	ns	↑
Wnt receptor signaling						
APC	Adenomatosis polyposis coli	2.00	1.63	0.41	ns	↑
CTNNB1	Catenin (cadherin-associated protein)	2.61	2.21	0.29	ns	↑
FRZB	Frizzled-related protein	1.60	1.30	0.52	ns	↑
FZD5	Frizzled homolog 5 (Drosophila)	1.67	1.16	0.48	ns	↑
FZD9	Frizzled homolog 9 (Drosophila)	1.52	1.18	0.61	ns	↑
WNT3	Wingless-type MMTV integration	1.82	1.60	0.60	ns	↑
WNT5B	Wingless-type MMTV integration	1.79	1.52	0.51	ns	↑
Protein-Tyrosin Phosphatase with potential role in tumorsupressor in colon						
PTPRJ	Protein tyrosine phosphatase	1.56	1.08	0.49	ns	↑
PTPN6	Protein tyrosine phosphatase	1.75	1.55	0.71	ns	↑
PTPN14	Protein tyrosine phosphatase	3.32	2.24	0.62	ns	↑
Cell Cycle Arrest-Regulation of cell cycle						
CCNA1	cyclin A	4.68	2.77	0.22	ns	↑
CDK6	cyclin-dependent kinase 6	5.72	6.32	0.13	ns	↑
CDK8	cyclin-dependent kinase 8	1.69	1.15	0.46	ns	↑
CDKN2B	Cyclin-dependent kinase inhibitor 2B	3.89	3.10	0.15	ns	↑
CDKN2D	Cyclin-dependent kinase inhibitor 2D	4.69	4.94	0.21	ns	↑
CGRRF1	Cell growth regulator	1.54	1.28	0.76	ns	↑
MAD2L2	MAD2 mitotic arrest deficient-like 2	1.55	1.05	0.59	ns	↑
MAPK12	Mitogen-activated protein kinase 12	1.76	1.17	0.39	ns	↑
MYC	V-myc myelocytomatosis oncogene	1.63	1.12	0.58	ns	↑
PTEN	Phosphatase and tensin homolog	2.12	1.57	0.30	ns	↑
TGFA	Transforming growth factor, alpha	1.71	1.21	0.47	ns	↑
TP53	Tumor protein p53	1.52	1.01	0.58	ns	↑
PIK3R1	Phosphatidyl Inositol 3-Kinase	1.52	1.19	0.55	ns	↑
PIK3R3	Phosphytidyl inositol 3-Kinase	1.55	1.15	0.55	ns	↑
Metal storage and utilization						
EPO	erythropoietin	5.39	3.31	0.19	ns	↑
FECH	Ferrochelatase (protoporphyria)	2.83	2.22	0.23	ns	↑
FTH1	Ferritin, heavy polypeptide 1	1.80	1.39	0.50	ns	↑
HFE	Hemochromatosis	2.66	2.24	0.27	ns	↑
SLC11A2	Solute carrier family 11 member 2	2.79	2.43	0.29	ns	↑
TF	Transferrin	1.61	1.26	0.58	ns	↑

Miscellaneous (several functions)						
ACO1	Aconitase 1, soluble	1.77	1.09	0.51	ns	↑
AHR	Arylhydrocarbon	1.87	1.25	0.43	ns	↑
ALAS1	Aminolevulinate, delta-, synthase 1	2.36	2.08	0.46	ns	↑
AR	Androgen Receptor	1.72	1.31	0.46	ns	↑
ARNT	aryl hydrocarbon receptor nuclear	3.74	3.57	0.19	ns	↑
ATF2	activating transcription factor 2	4.78	3.06	0.16	ns	↑
ESR1	estrogen receptor 1 (ER alpha)	2.61	1.63	0.40	ns	↑
ESR2	estrogen receptor 2 (ER beta)	2.24	1.54	0.29	ns	↑
HPX	Hemopexin	2.25	1.53	0.30	ns	↑
TRAF1	TNF receptor-associated factor 1	4.17	3.18	0.21	ns	↑
UBE2D1	Ubiquitin-conjugating enzyme	2.01	1.69	0.45	ns	↑
IKBB	Kappa Beta Thyroid Receptor Protein	1.85	1.66	0.97	ns	↑
IAP1	Baculoviral IAP Repeat	2.77	2.26	0.20	ns	↑
BIRC6	Baculoviral IAP Repeat	3.36	3.17	0.31	ns	↑
BIRC7	Baculoviral IAP Repeat	2.93	1.09	0.26	ns	↑

Appendix E.8 Genes down regulated by FB in LT 97 cells

Gene name	Descriptions	Ratio	SD	Pvalue	Sign	
Phase II						
Acetyltransferases						
ACAT2	Acetyl-Coenzyme A	0.39	0.20	0.01	*	↓
Epoxide Hydrolases						
EPHX2	Epoxide hydrolase 2	0.51	0.28	0.04	*	↓
Methyltransferases						
COMT	Catechol-Omethyltransferase	0.37	0.13	0.05	ns	↓
Stress and Signal transduction						
Oxidative or Metabolic Stress						
GPX1	Glutathione peroxidase 1	0.52	0.20	0.12	ns	↓
DNA Damage and Repair						
XRCC5	X-ray repair complementing	0.56	0.13	0.17	ns	↓
Growth Arrest and Senescence						
PCNA	Proliferating cell nuclear antigen	0.45	0.30	0.06	ns	↓
Cell Cycle Arrest-Regulation of cell cycle						
CCNB1	cyclin B1	0.54	0.24	0.12	ns	↓
CDK4	cyclin-dependent kinase 4	0.50	0.19	0.07	ns	↓
CDK1	Cell Division Control Protein 2	0.59	0.17	0.11	ns	↓
Positive controls (Housekeeping)						
TUBB1	Tubulin, beta 1 and 5	0.42	0.18	0.13	ns	↓
Miscellaneous (several functions)						
MCM7	minichromosome maintenance deficient	0.28	0.14	0.02	*	↓
TYMS	Thymidylate synthetase	0.35	0.08	0.00	***	↓

10 Curriculum vitae

Personal Details:

Name	Umang Munjal
Maiden name	Umang Miglani
Date of Birth	26th March 1977
Birth place	Hisar, India
Nationality	Indian
Sex	Female
Family stand	Married, one daughter
Present Address	Im Lohr 9, 68199 Mannheim, Germany.
Permanent address	House no 972, Sector 13 P Hisar, Haryana, India
Mobile	+49-17627412780
E-Mail Address	umangmiglani@hotmail.com, b5muum2@uni-jena.de

ACADEMIC PROFILE:

October 2005 - till date: Institute of Nutrition, Department of Nutritional Toxicology, Friedrich-Schiller-University Jena, Dornburger Str. 24, D-07743Jena, Germany
Title / Position: PhD student

April 2005 to September 2005: German language course at the inter-DaF eV am Herder Institut der Universität Leipzig.

February 2003 to August 2003: Institute of Biochemistry, School of Veterinary medicine, Bunteweg 17, D-30559, Hannover, Germany
Title / Position: Guest Student

August 2003 to March 2005: Pregnancy leaves

January 2001 to November 2002: Department of Veterinary Physiology, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttaranchal 263145, India.
Title / Position: M.V.Sc Scholar

September 1995 to December 2000: Bachelor's degree in Veterinary sciences and Animal husbandry: College of Veterinary Science, Hisar Haryana-125001, India

AWARDS/ FELLOWSHIPS:

1. Recipient of "DAAD fellowship" for pursuing PhD at Institute of Nutrition, Department of Nutritional Toxicology, Jena, Germany.
2. Recipient of "ICAR Junior Research Fellowship" for pursuing two year M.V.Sc degree at College of Veterinary Sciences, Division of Physiology, GB Pant University of Agriculture and Technology, Pantnagar, India.
3. Best Poster Award, October 2002 by Indian Society for Veterinary Pharmacology and Toxicology

MEMBERSHIP OF PROFESSIONAL SOCIETIES:

1. Member of Society of Animal Physiologists of India (SAPI)
2. Life Member of Society of Animal nutrition
3. Member of Veterinary Council of India (VCI)
4. Student member of European Association of Cancer Research

PUBLICATIONS:

1. **Umang Miglani**, S. K. Rastogi., J. P. Korde and Pradeep Kumar **(2002)**. Experimental Studies on Electrocardiographic changes in rats subjected to heat stress. Abstract published in III Annual Conference of Indian Society for Veterinary Pharmacology and Toxicology on 24-26 Oct 2002 at Pantnagar, India.
2. **Umang Miglani**, S. K. Rastogi., J. P. Korde. and J. L. Singh **(2005)**. Electrocardiographic changes associated with feed, water restriction and heat exposure in domestic goats**. Indian J. of Vet. Med**. 25(2) 85-88.
3. **Umang Miglani**, S.K. Rastogi, J.P. Korde and Pardeep Kumar **(2007)**. Physiological and haematological responses of goats to feed, water restriction and heat exposure. **Indian Journal Small Ruminants** 13(1): 105-108.
4. Umang Munjal **and S.K. Rastogi**. Influence of environmental temperature on electrocardiographic and physiological responses in adult Wistar rats. **Indian Journal of Animal Physiology** (accepted)
5. Glei, M, Hofmann, T, **Munjal, U,** Scharlau, D, Pool-Zobel, BL. **(2007)** Chemopreventive potential of dietray fibre-*in vitro* investigations with human colon cells". Abstract published in **Brazilian Journal of Toxicology**, Volume 20; Suppl. 2.
6. Beatrice L.Pool Zobel, Nina Habermann, Thomas Hofmann, Stefanie Klenow, Yvonne Knobel, Claudia Miene, **Umang Munjal**, Daniel Scharlau, Selvaraju Veeriah, Michael Glei **(2007)** Modulation of carcinogenesis by food components:targets and mechanisms of action. Abstract published in **Ann Nutr Metab**; 51(supp 1): Page 15
7. **U. Munjal**; D. Scharlau and B.L. Pool-Zobel **(2008)** Fermentation products of inulin-type fructans reduce proliferation and induce apoptosis in human colon

tumor cells of different stages of carcinogenesis. Abstract published in **European Journal of Cancer supplements** Volume 6, Issue 3, March 2008, Page 55

8. **Umang Munjal, Michael Glei, Beatrice Louise Pool-Zobel, Daniel Scharlau (2009)** Fermentation products of inulin-type fructans reduce proliferation and induce apoptosis in human colon tumor cells of different stages of carcinogenesis. **British Journal of Nutrition (accepted)**
9. **Umang Munjal,** Daniel Scharlau, Michael Glei Fermentation products of inulin type fructans enhance toxicological defence by favourably modulating expression of genes related to oxidative stress and biotransformation in human adenoma and carcinoma cell lines, **in preparation**
10. **Umang Munjal,** Daniel Scharlau, Michael Glei Potential of fermentation products of inulin-type fructans to modulate the expression of apoptosis relevant genes in (LT97) and tumor (HT29) cells, **in preparation**

POSTER PRESENTATION

1. **Umang Miglani**., S. K. Rastogi., J. P. Korde. and Pradeep Kumar **(2002)**. Experimental Studies on Electrocardiographic changes in rats subjected to heat stress. Abstract published in III Annual Conference of Indian Society for Veterinary Pharmacology and Toxicology on 24-26 Oct 2002 at Pantnagar, India
2. **U. Munjal,** D. Scharlau, B.L. Pool-Zobel **(2007)** Fermentation products of inulin-type fructans have chemoprotective activities by protecting from H_2O_2-caused DNA damage and suppressing survival of colon carcinoma HT29 cells presented at "44.Wissenschaftlicher Kongress der DGE" on 8th March 2007 Halle, Germany
3. **U. Munjal,** D. Scharlau, B.L. Pool-Zobel **(2007)** Comparison of effects on secondary chemoprevention of fermentation products of inulin-type fructans in human colon adenoma (LT97) and carcinoma (HT29) cell lines presented at "5th Indo-German workshop on July 12-14, 2007 in DKFZ Heidelberg Germany
4. **U. Munjal,** D. Scharlau, B.L. Pool-Zobel **(2008)** Fermentation products of inulin-type fructans reduce proliferation and induce apoptosis in human colon tumor cells of different stages of carcinogenesis presented at 5th International conference "Cancer prevention 2008" on March 6-8 2008 in St Gallen Switzerland Oncology conference
5. **Munjal,** D. Scharlau, S. Haugvik, M. Glei, B.L.Pool-Zobel **(2008)** Fermentation products of dietary fibres enhance toxicological defence by favourably modulating expression of genes related to oxidative stress and biotransformation in HT29 human colon carcinoma cell line presented at 3rd ESF Meeting on October 1-4 2008 in Innsbruck Austria

11 Acknowledgements

This work was carried out during the years 2005-2008 at the Department of Nutritional Toxicology, Institute of Nutrition, Friedrich Schiller University, Jena, Germany. Late Professor Pool Zobel supervised the work initially, encouraging and supporting, provided the working facilities in her group and introduced me to the field of research. I want to warmly thank Late Prof. Pool Zobel who fought hard against her deadly disease and will be always missed.

I wish to express my gratitude for PD Dr. Michael Glei, who has been following the progress of this thesis work from the beginning through his energetic approach, ideas, technical knowledge, optimism and motivation. I consider myself lucky to have been inspired by his curiosity in exploring the scientific questions.

I owe my thanks to Dr. Daniel Scharlau for investing his time in carefully and patiently evaluating the thesis. His comments and advices were invaluable from the beginning till the end of this study. I admire his scientific objectivity and wish to thank him for the guidance.

I want to thank Frau Esther Woschee for being a helping hand, technical guide, moral support, good friend and helping me out in the early years of work-thank you.

I want to acknowledge my fellow colleagues Anne, Franzi, Wiebke, Stefanie, Thomas, Nina, Claudia, Steffi, Raju, Julia, Kathrin, Anke and others, who have joined our lab for longer and shorter periods of time for their support and discussions. I also thank all the members of Department of Nutritional Toxicology; Kerstin, Edda and Ines who

have created a pleasant working environment and provided technical help whenever I needed.

For Secretarial assistance I am grateful to Claudia Lüdtke and Anke Partschefeld. This study was financially supported by DAAD.

My warmest thanks goes to my parents Mrs Sudesh and Mr. N.P. Miglani, brothers Vibhor and Puneet, sister in law Teena, parents in law Mrs Rekha and Mr. R.N. Munjal, sister and brother in law Mrs Anu and Mr. Yash Mehta and their children Manivek and Sahil for their loving support. All my friends, who have encouraged me in my work during the years, especially Pratibha, and whole Joshi family are sincerely thanked.

To my husband Sarvan and my daughter Nishka all I can say is it would take another thesis to express my deep love for you. Your patience, love and encouragement have upheld me, particularly in those many days in which I spent more time with my computer than with you. Both of you are the precious gems of my life. No more doctorates I promise! now it's your turn.

Jena, April 14, 2009

Certification of Originality

To the best of my knowledge and belief, this thesis does not contain any material previously submitted for a degree or diploma in any university or any material previously written or published by any other person, except where due acknowledgment is made in the text.

Jena, April 14, 2009 (Umang Munjal)